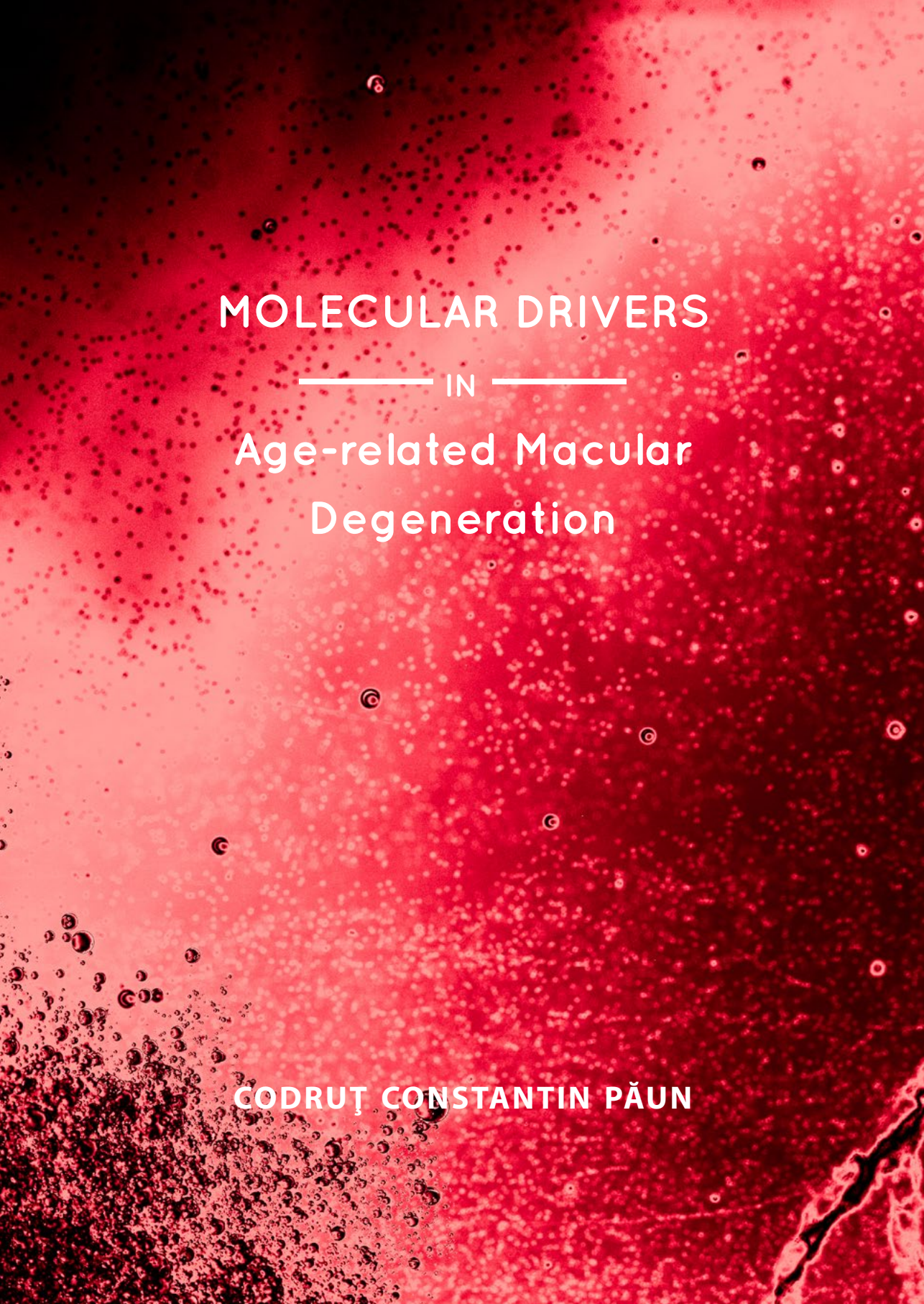
MOLECULAR DRIVERS
IN
Age-related Macular Degeneration
CODRUŢ CONSTANTIN PĂUN

Molecular Drivers

— IN —

Age-related Macular Degeneration

CODRUŢ CONSTANTIN PĂUN

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Molecular drivers in age-related macular degeneration

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to obtain the degree of doctor
from Radboud University Nijmegen
on the authority of the Rector Magnificus prof. dr. J.H.J.M. van Krieken,
according to the decision of the Council of Deans
to be defended in public on Thursday, December 6, 2018
at 10.30 hours

by

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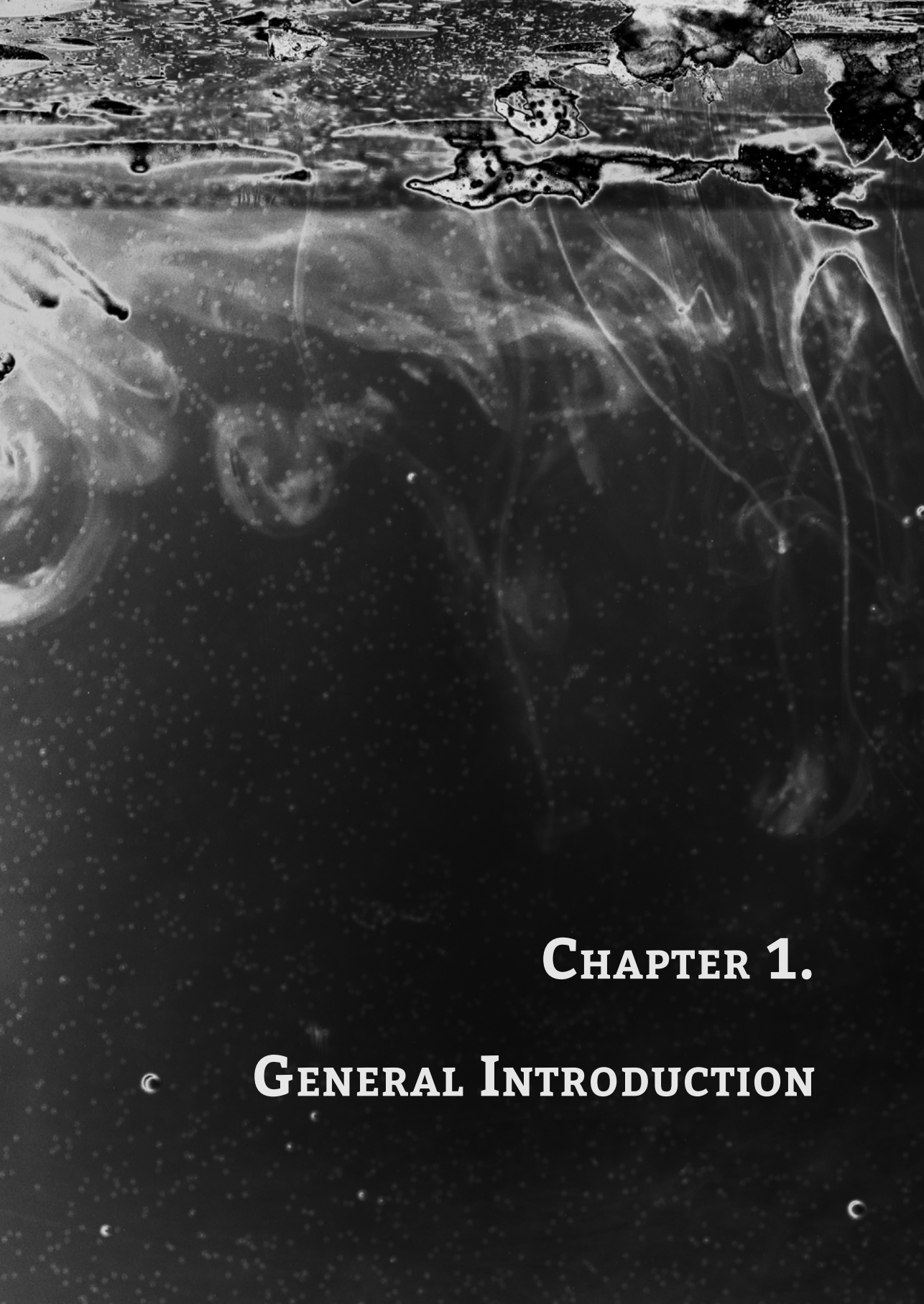

Chapter 1.

General Introduction

1.1 THE RETINA

The retina is the light-sensitive inner lining of the eye, which is responsible for vision (Figure 1A). A radial section of the retina reveals its highly organized multilayered structure (Figure 1B).

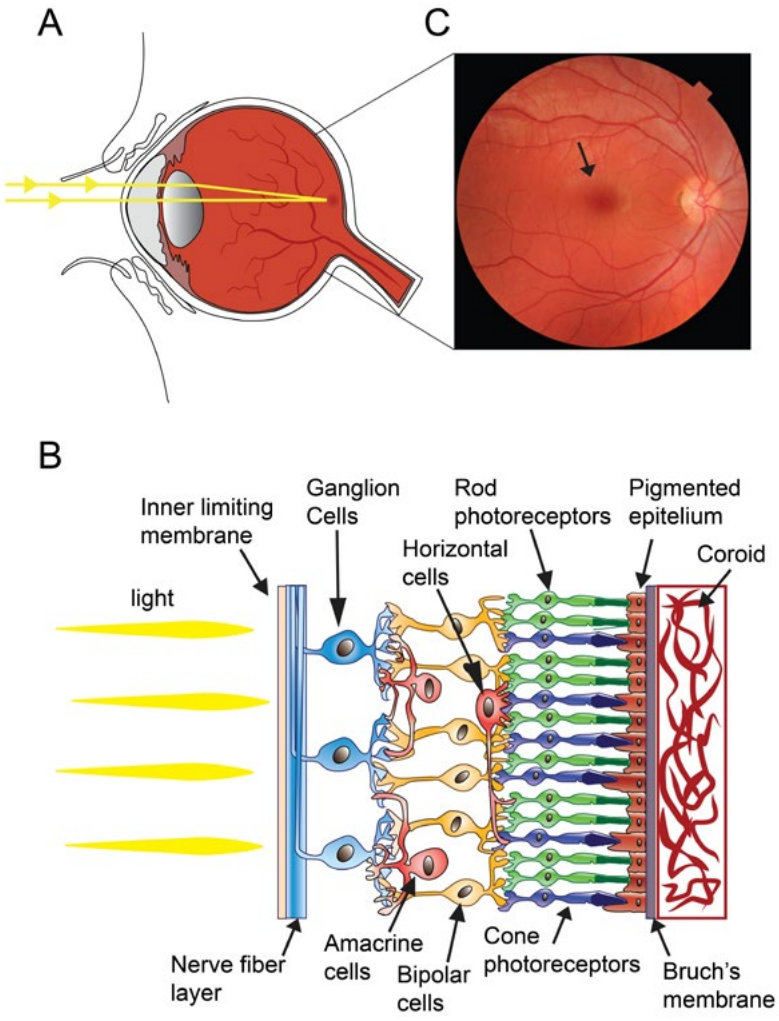

Figure 1. The retina. A. Schematic representation of the eye. **B.** Schematic representation of a cross-section of the retina. The light that enters the eye passes through all the layers, starting with the inner limiting membrane, before reaching the rod and cone photoreceptors where signal transduction is initiated. Retinal cells layers are annotated in the figure. **C.** fundus photograph of a healthy human retina with the black arrow indicating the macula.

Upon light stimulation, the phototransduction cascade in the photoreceptor cells is initiated, resulting in a signal to the brain. Underneath the neurosensory retina lies a single layer of highly specialized cells: the retinal pigment epithelium (RPE). RPE cells serve multiple crucial functions, such as maintaining photoreceptor homeostasis by facilitating the removal of cellular and metabolic waste from the neurosensory retina to the underlying vasculature, the choroid, and the diffusion of nutrients from the choroid to the retina.

The RPE is separated from the underlying choroid by Bruch's membrane, an extracellular matrix structure comprised of the RPE basement membrane, an inner collagenous zone, central elastin, an outer collagenous zone and the choriocapillaris basement membrane.

A color photograph of the inner lining of the eye shows an area in the center of the retina called macula lutea (Latin for yellow spot; Figure 1C). The macula is responsible for central, high-resolution vision as well as color vision under daylight conditions due to the high density of cone photoreceptors located in this area. In contrast, in the peripheral retina the rod photoreceptors that specialize in contrast detection predominate, enabling vision in low light conditions.

1.2 AGE-RELATED MACULAR DEGENERATION

Age-related macular degeneration (AMD) is the leading cause of irreversible vision loss in elderly of European decent. It is projected that 196 million people will be affected worldwide with any form of AMD in the year 2020, and due to aging of the population this number will increase to 288 million by 2040.[1] AMD is a progressive disease that causes gradual loss of central vision as a result of degenerative processes that occur in the macula.

An important hallmark of the disease is the formation of extracellular deposits called drusen, found between the RPE and the inner collagenous zone of Bruch's membrane, which can be readily observed upon ophthalmological examination. The number, size and location of drusen, together with the presence or absence of pigmentary changes are phenotypic characteristics that are used to categorize the early phases of the disease into different stages. Progression to late stage disease can lead to two forms:

dry AMD or geographic atrophy (GA), where widespread cell death underlies vision loss, and wet AMD, characterized by choroidal neovascularization (CNV).

1.3 AMD IS A MULTIFACTORIAL DISEASE

AMD is a multifactorial disorder caused by a combination of genetic and environmental factors. The two strongest environmental factors that increase risk for AMD are age and smoking. Pooled data from three continents showed that AMD prevalence increases dramatically with age, from 0.2% in individuals 55-64 years old to 13% in individuals above 85 years.

Independent of the risk given by older age, smoking adds a 2-4 fold higher risk towards AMD [2].

Nutritional factors have also been linked to AMD. Increased dietary fat intake and obesity were positively associated with increased risk for AMD while a protective effect for antioxidants, nuts, fish, and omega-3 polysaccharide unsaturated fatty acids has been described [3-5]. The association with smoking and the protective effect of antioxidants suggests that oxidative stress contributes to the pathogenesis of AMD.

Evidence that AMD has a strong genetic component comes from early familial and twin studies [6-10], and the heritability of AMD has been assessed to be as high as 45% [11]. Since then, a series of genome-wide association studies (GWAS) have attempted to unravel the genetic components contributing to the disease. In a GWAS, commonly occurring single nucleotide polymorphisms (SNPs) that are spread across the genome are tested for association with a (disease) trait. In AMD, several GWASes had been performed [12-25], but it became clear that only part of the genetic contribution to disease could be explained through this approach. It was hypothesized that rare or low frequency genetic variation could fill that gap, and several reports have linked rare variants in a number of genes to AMD [26].

Using an exome chip, a microarray that contains 264,655 common variants and 174,695 low frequency or rare variants, a very large GWAS was recently conducted[27]. In this study, using 16,144 cases and 17,832 control individuals, 34 genomic loci were identified to be associated with AMD, explaining between 18 to 34% of the disease

liability. At the 34 loci, 52 variants were identified that are independently associated with AMD, of which 45 were common genetic variants and 7 were low frequency variants [27].

Because of the inherent low occurrence of rare genetic variants (< 1% allele frequency), it is challenging to obtain statistical significance in association studies for individual variants, unless very large sample sizes are available. Alternatively, burden tests have been developed to test for an accumulation of (rare) genetic variation in one gene, or a group of genes that constitute a pathway in cases compared to controls. In AMD, so far, only the *CFH*, *CFI*, *SLC16A8* and *TIMP3* genes have shown a burden of rare variants compared to controls [27,28]

Although 34 independent genomic loci have been identified in the largest genetic study in AMD to date, from a biological perspective, these loci are far from being independent. The genes in these AMD-associated loci can be clustered into four major biological pathways: the complement system, lipid metabolism, extracellular matrix (ECM) remodeling and angiogenesis signaling.

1.4 COMPLEMENT SYSTEM

The first association identified in a GWAS study in AMD was with a SNP in the *CFH)* gene[12]. This association remained strongly associated with AMD as subsequent GWA studies increased in size [15,25,27], and additional genes encoding components of the complement cascade emerged (Table 1).

The complement system is a complex integral part of our innate immunity, with a multitude of functions that extend beyond simple danger elimination. The complement system acts as the first line of defense against foreign intruders and it also complements the rest of the immune system by orchestrating immunological and inflammatory processes [29]. Besides its well-known role of host defense against foreign intruders [30] the complement system is recognized to participate in diverse processes such as clearance of immune complexes, angiogenesis, tissue regeneration and lipid metabolism [31]. With such a wide range of functions, the complement system is tightly regulated to discriminate between healthy host tissue, cellular debris, apoptotic cells and foreign intruders. This is achieved by maintaining a balance between activation

Table 1. Variants in or near genes encoding component of the complement system, found to be associated with AMD at genome wide level ($p<5x10^{-8}$).

No.	Locus name	Index variant	Major / minor allele	Minor allele frequency		Association results	
				Cases	Controls	OR	P
1	*CFH*	rs10922109	C/A	.223	.426	0.51	1.0×10^{-131}
2	*CFH*	rs570618	G/T	.580	.364	1.74	9.2×10^{-76}
3	*CFH*	rs121913059	C/T	.003	.00014	47.63	2.2×10^{-35}
4	*CFH*	rs148553336	T/C	.003	.009	0.31	8.8×10^{-17}
5	*CFH*	rs187328863	C/T	.054	.028	1.47	2.8×10^{-12}
6	*CFH (CFHR3/CFHR1)*[b]	rs61818925	G/T	.284	.385	1.18	6.3×10^{-9}
7	*CFH*	rs35292876	C/T	.021	.009	1.54	9.5×10^{-8}
8	*CFH*	rs191281603	C/G	.007	.006	0.41	7.7×10^{-7}
9	*CFI*	rs10033900	C/T	.511	.477	1.15	1.2×10^{-13}
10	*CFI*	rs141853578	C/T	.003	.0008	5.12	7.4×10^{-12}
11	*C9*	rs62358361	G/T	.016	.009	1.67	7.2×10^{-9}
12	*C2/CFB/SKIV2L*	rs116503776	G/A	.090	.148	0.51	5.0×10^{-96}
13	*C2/CFB/SKIV2L*	rs144629244	G/A	.016	.012	2.79	1.0×10^{-32}
14	*C2/CFB/SKIV2L (PBX2)*[b]	rs114254831	A/G	.284	.260	1.13	8.8×10^{-9}
15	*C2/CFB/SKIV2L*	rs181705462	G/T	.018	.012	1.56	2.8×10^{-8}
16	*TMEM97/VTN*	rs11080055	C/A	.463	.486	0.92	1.5×10^{-5}
17	*C3*	rs2230199	C/G	.266	.208	1.47	1.6×10^{-60}
18	*C3*	rs147859257	T/G	.012	.004	3.22	4.1×10^{-26}
19	*C3 (NRTN/FUT6)*[b]	rs12019136	G/A	.036	.048	0.74	4.0×10^{-9}

Table was adapted from [27]

and inhibition [29]. The complement system is composed of three pathways: the classical pathway (CP), the lectin pathway (LP) and the alternative pathway (AP), which converge at the level of complement component 3 (C3) [32]. The process continues through a series of enzymatic reactions that finally lead to the formation of the membrane-attack-complex (MAC) [33]. A schematic representation of the complement system is presented in Figure 2.

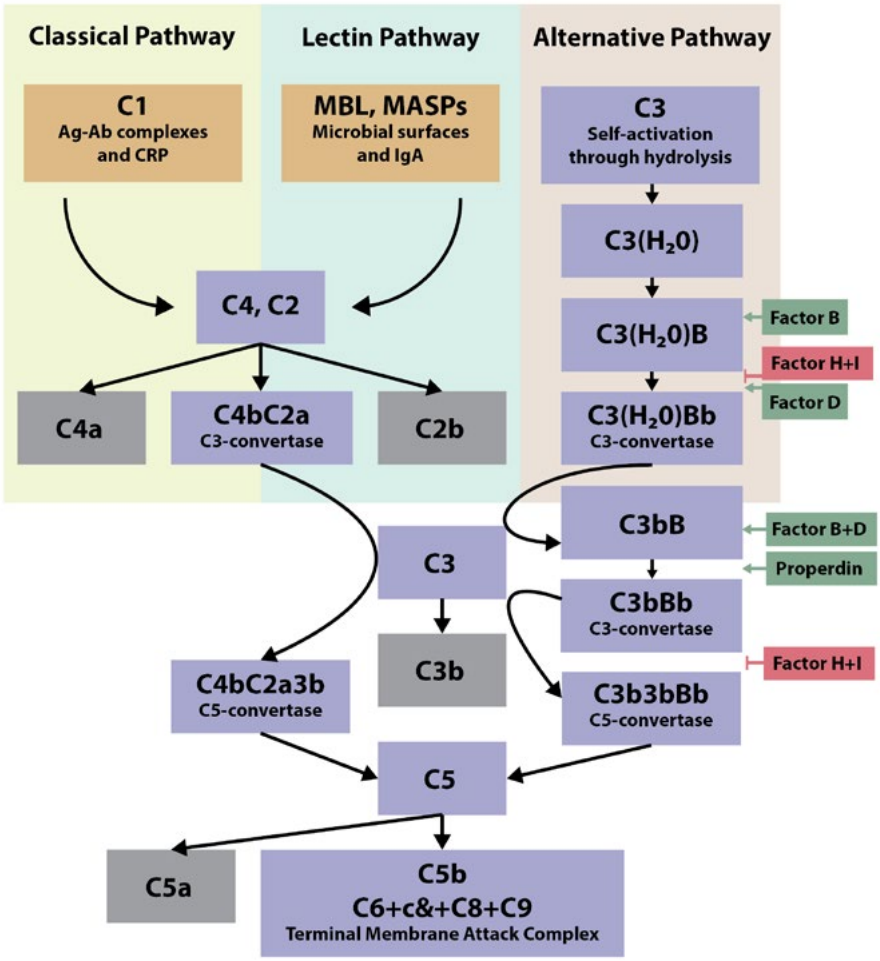

Figure 2. Schematic overview of the complement system. Each pathway of the complement system has a different way of activation. Only the AP self-activates in a process known as tick-over, where the spontaneous hydrolysis of C3 to a bioactive form, C3(H_2O), which binds complement factor B (FB), and subsequently is cleaved by complement factor D (CFD) into C3(H_2O)Bb, thus forming the initial C3 convertase of the alternative complement pathway. This maintains a low level of continuous activation, which is essential for pathogen monitoring [34]. The CP is activated by IgM/IgG clusters, but also by endogenous pattern recognition molecules and by recognizing distinct structures directly on microbial and apoptotic cells [29]. The LP is activated by mannose-binding lectin and ficolins that recognize carbohydrate patterns [35]. The three complement pathways converge after the cleavage of C3 into C3a and C3b and the subsequent formation of the C5 convertase complexes, which in turn cleave C5 into C5a and C5b, initiating the final steps of the complement cascade. C5b together with C6, C7, C8, and C9 form the MAC, which creates pores in the membrane lipid bilayer, thus resulting in pathogen or cell destruction [36].

Deregulations and deficiencies within the complement system have been reported to be associated with numerous inflammatory diseases and autoimmune disorders [37]. In AMD, the association of the complement system is not limited to genes only [38-41]. Starting with the work of Van der Schaft [42] in the early '90s, multiple studies have identified complement activators, components and regulatory proteins in drusen of AMD patients [43]. In addition to this, complement activation has been observed in AMD patients, both systemically and in the eye [40,44,45], but these activation patterns are highly variable and large groups of patients and controls are needed to obtain statistical significance. Thus, genetic, histopathological and physiological evidence firmly supports the notion that aberrant complement regulation is involved in AMD.

1.5 OTHER PATHWAYS INVOLVED IN AMD PATHOGENESIS: LIPID METABOLISM, OXIDATIVE STRESS, ECM REMODELING, AND ANGIOGENESIS

1.5.1 Lipid metabolism

A second major pathway associated with AMD is the lipoprotein/lipid metabolism pathway. Variants in four genes encoding components of the lipid metabolism have been associated with AMD (Table 2.) [27]. Besides the genetic associations, involvement of lipoproteins in AMD comes from evidence that lipids and apolipoproteins make up more than 40% of drusen volume [46]. Systemic levels of components belonging to this pathway, such as HDL and LDL, have been extensively studied in AMD with no clear consensus on the exact contribution of these particles in the disease process [47,48]. Many conflicting results have been published, but when taken together, larger and thus a more statistically powered study implicated the HDL particle as a risk conferring entity in AMD [49].

1.5.2 Oxidative stress

During aging, accumulated effects of environmental stresses put a strain on multiple systems, including the cells and tissues of the eye. One of the major outcomes of these accumulated effects is thought to be an increased vulnerability to oxidative stress, to which the retina is particularly sensitive because of its high oxygen demand. With aging mitochondrial oxidation is impaired and oxidative damage is widely observed [50-52]. Oxidative damage has been reported in AMD [53], and patients' RPE cells display higher reactive oxygen species (ROS) production and lower mitochondrial activity [54]

Table 2. Variants in or near genes encoding components of the lipid metabolism, reported to be associated with AMD at genome wide level ($p<5x10^{-8}$)

No.	Locus name	Index variant	Major / minor allele	Minor allele frequency		Association results	
				Cases	Controls	OR	P
1	*ABCA1*	rs2740488	A/C	.255	.275	0.89	6.0×10^{-7}
2	*LIPC*	rs2043085	T/C	.350	.381	1.15	7.7×10^{-13}
3	*LIPC*	rs2070895	G/A	.195	.217	0.86	1.8×10^{-10}
4	*CETP*	rs5817082	C/CA	.232	.264	0.87	2.7×10^{-8}
5	*CETP*	rs17231506	C/T	.348	.315	1.11	1.2×10^{-6}
6	*APOE*	rs429358	T/C	.099	.135	0.67	3.9×10^{-39}
7	*APOE(EXOC3L2/MARK4)*[b]	rs73036519	G/C	.284	.302	0.91	2.4×10^{-5}

Table was adapted from [27]

Table 3. Variants in or near genes encoding proteins involved in ECM remodeling, found to be associated with AMD at genome wide level ($p<5x10^{-8}$)

No.	Locus name	Index variant	Major / minor allele	Minor allele frequency		Association results	
				Cases	Controls	OR	P
2	*COL4A3*	rs11884770	C/T	.258	.278	0.92	2.6×10^{-4}
3	*ADAMTS9-AS2*	rs62247658	T/C	.466	.433	1.14	7.8×10^{-11}
4.1	*COL8A1*	rs140647181	T/C	.023	.016	1.85	1.6×10^{-14}
4.2	*COL8A1*	rs55975637	G/A	.132	.117	1.16	3.8×10^{-7}
17	*ARHGAP21*	rs12357257	G/A	.243	.223	1.12	1.8×10^{-6}
18	*ARMS2/HTRA1*	rs3750846	T/C	.436	.208	2.93	6.0×10^{-645}
19	*RDH5/CD63*	rs3138141	C/A	.222	.207	1.18	4.7×10^{-8}
21	*B3GALTL*	rs9564692	C/T	.277	.299	0.90	1.0×10^{-6}
25	*CTRB2/CTRB1*	rs72802342	C/A	.067	.080	0.79	8.0×10^{-9}
29	*CNN2*	rs67538026	C/T	.460	.498	0.90	1.4×10^{-6}
31	*MMP9*	rs142450006	TTTTC/T	.124	.141	0.84	5.3×10^{-9}
33	*SYN3/TIMP3*	rs5754227	T/C	.109	.137	0.79	5.7×10^{-16}
34	*SLC16A8*	rs8135665	C/T	.217	.195	1.14	1.4×10^{-8}

Table was adapted from [27]

compared to controls. Because the eye, and especially the macula, is susceptible to pathological oxidative processes affecting proteins and lipids, multiple factors related to oxidative stress have been the subject of research and found to be associated with AMD [55-58].

1.5.3 Extracellular matrix (ECM) remodeling

Genetic evidence also suggests that ECM remodeling is involved in the disease process. Drusen form within Bruch's membrane, which is layered sheet of ECM [59]. Moreover, genetic variation in genes encoding proteins involved in ECM remodeling, such as matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases, are associated with AMD [27] (Table 3). These findings suggest that tissue remodeling, potentially in the context of an immunological response with infiltrating immune cells, contributes to the etiology of the disease.

1.5.4 Angiogenesis

The formation of new blood vessels from the choroid and growth into the retina is central to AMD pathology in the late (wet) stage. Upon a hypoxic cue, the choroid begins to develop fragile new blood vessels to oxygenize the retina. The main driver of this effect is vascular endothelial growth factor (VEGF). The gene coding for this molecule is also genetically associated with the disease [27], but more importantly, when VEGF is targeted using antibodies, the neovascular disease process is arrested, resulting in an improved visual outcome in many patients with the wet form of AMD [60-62].

1.6 TREATMENT FOR AMD

To date, only people that suffer from wet AMD have an approved therapy available to them, targeting VEGF with monthly intravitreal injections. These patients represent the minority, as only between 10%-15% of patients progress to this form of AMD [63]. Patients with early, intermediate or the GA forms of AMD do not have a treatment available to them. Although AREDS2 supplementation can slow down the progression of AMD [64], no definitive therapy exists to arrest disease progression definitively.

Based on all the available evidence, targeting of the complement system is becoming an emerging alternative strategy for the treatment of AMD. Several clinical trials

targeting components of the complement system (C3, C5, and FD), have been performed or are currently ongoing [36,65,66]. The outcomes of these trials showed limited [67] or no meaningful effects [NCT01603043] [68]. It is not well understood why targeting the complement system was not very effective so far, but it may be a result of the high variability in complement activation in both patients and controls.

1.7 AIMS AND OUTLINE OF THE THESIS

The aim of this thesis is to understand the molecular drivers of AMD, and to study the functional consequences of genetic variation associated with AMD.

Chapter 2 summarizes all reported findings of compounds measured in systemic and eye fluids, in relation to AMD. This literature review represents the first systematic categorization of potential biomarkers for AMD and their use in research, diagnostics and clinical trials.

Chapter 3 explores rare variants in the *C3* gene of the complement system. This strategy was developed in an attempt to solve part of the missing heritability in AMD.

Chapter 4 describes the association of AMD-associated variants with systemic complement activation to identify how these variants influence complement activation levels.

Chapter 5 advances the work in Chapter 4 by combining the effects of several variants in genes encoding components in the complement system into a complotype. Here, we examined the combined effect of multiple genetic variants and identified a novel complotype combination, which is associated with both AMD and systemic complement activation.

Chapter 6 describes a statistical analysis of AMD-associated variants and complement activation levels with multiple factors belonging to the lipid metabolism pathway. As multiple pathways are involved in the pathogenesis of AMD, this chapter explores where these pathways intersect and what the relevance of this intersection is.

Chapter 7 takes a non-biased approach using GWAS to identify genetic variants

that influence complement activation levels. In the future, such genetic markers can potentially help stratify patients into subgroups that could benefit the most from novel treatments targeting the complement system in AMD.

Chapter 8 discusses the results of the previous chapters, and places them in the context of current literature and advancements in the field.

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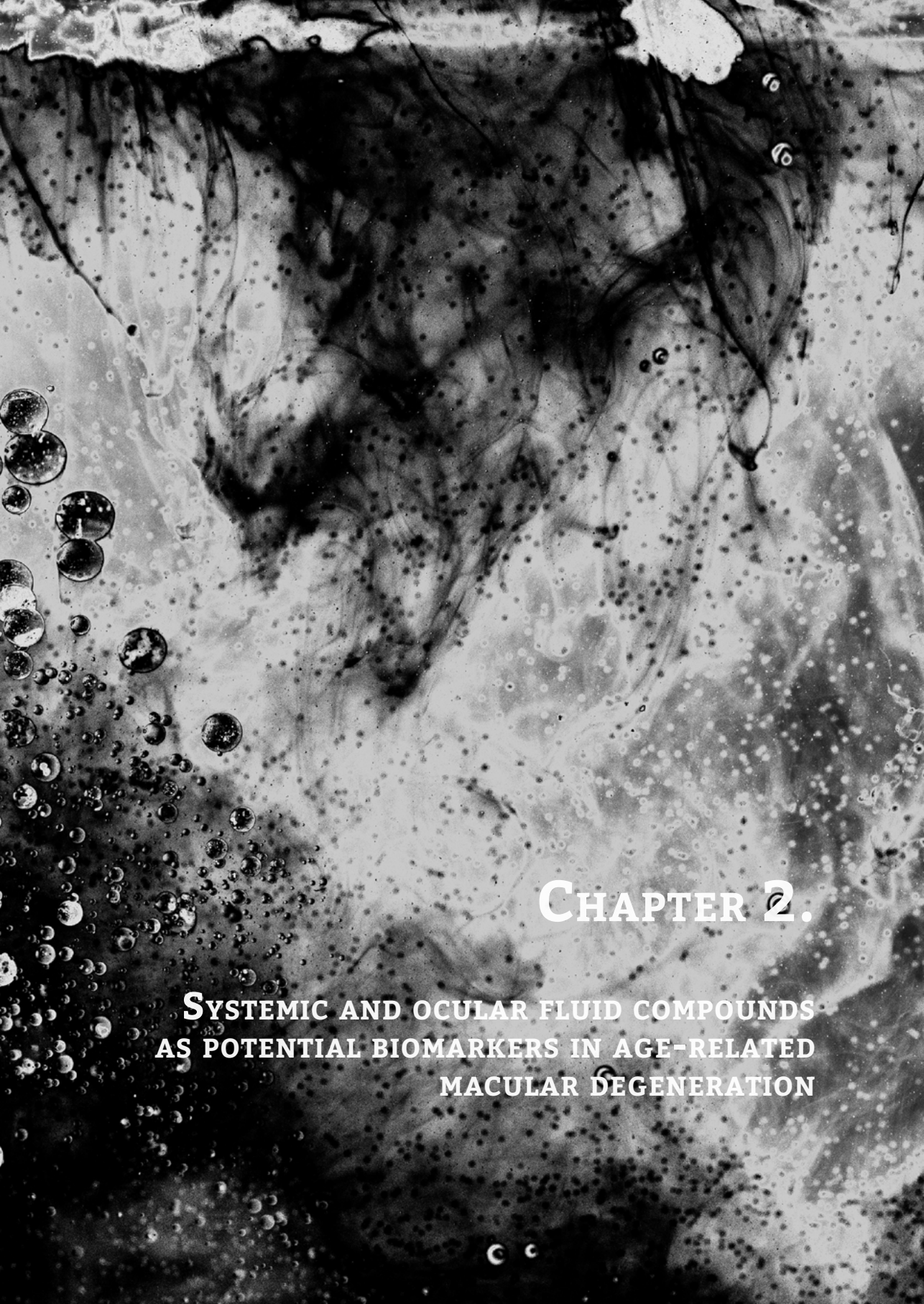

Chapter 2.

Systemic and ocular fluid compounds as potential biomarkers in age-related macular degeneration

Adapted from

Systemic and ocular fluid compounds as potential biomarkers in age-related macular degeneration

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ABSTRACT

Biomarkers can help unravel mechanisms of disease and identify new targets for therapy. They can also be useful in clinical practice for monitoring disease progression, evaluation of treatment efficacy, and risk assessment in multifactorial diseases, such as age-related macular degeneration (AMD). AMD is a highly prevalent progressive retinal disorder for which multiple genetic and environmental risk factors have been described, but the exact etiology is not yet fully understood. Many compounds have been evaluated for their association with AMD. We performed an extensive literature review of all compounds measured in serum, plasma, vitreous, aqueous humor, and urine of AMD patients. Over 3600 articles were screened, resulting in more than 100 different compounds analyzed in AMD studies, involved in neovascularization, immunity, lipid metabolism, extracellular matrix, oxidative stress, diet, hormones, and comorbidities (such as kidney disease). For each compound, we provide a short description of its function and discuss the results of the studies in relation to its usefulness as AMD biomarker. In addition, biomarkers identified by hypothesis-free techniques, including metabolomics, proteomics, and epigenomics, are covered. In summary, compounds belonging to the oxidative stress pathway, the complement system, and lipid metabolism are the most promising biomarker candidates for AMD. We hope that this comprehensive survey of the literature on systemic and ocular fluid compounds as potential biomarkers in AMD will provide a stepping stone for future research and possible implementation in clinical practice.

2.1 INTRODUCTION

The term biomarker refers to an objective, measurable characteristic that is indicative of a biological process (normal, pathogenic, or in response to treatment).[30] Biomarkers can help unravel mechanisms of disease and identify (new) targets for treatment. The potential benefit of biomarkers in drug development is to allow earlier, more robust drug safety and efficacy measurements.[385] In addition, biomarkers can be useful in clinical practice for detecting disease, monitoring disease progression, evaluation of treatment efficacy, and risk assessment. Biomarker testing is an important step toward personalized medicine in many diseases, such as cancer,[198] but also in age-related macular degeneration (AMD).

AMD is the leading cause of irreversible loss of vision among the elderly in the Western world, and the prevalence of AMD is expected to increase with population ageing.[364]

The early stage of AMD is characterized by subretinal yellowish deposits, known as drusen, and changes in macular pigmentation.[43, 151] At this stage, patients usually express little or no complaints. As AMD progresses, central vision becomes increasingly blurred, resulting in irreversible vision loss in the advanced stages of the disease. Two subtypes of advanced AMD can be distinguished: geographic atrophy (GA) and neovascular AMD (nAMD).[43, 151] The atrophic form of AMD is characterized by cell death of the retinal pigment epithelium (RPE) and photoreceptors causing gradual vision loss.[136] Neovascular AMD, also referred to as "wet" or "exudative" AMD, is characterized by abnormal vessel growth into the retina from the choroid (choroidal neovascularization [CNV]). Leakage from these fragile neovascularizations can cause rapid loss of vision.[363] In this review, we will use the following terms for the different AMD subgroups described in literature: any AMD, early AMD, advanced AMD (GA/neovascular/any advanced), and dry AMD (for definitions of these terms, see Table 1).

AMD is a multifactorial disease, and many risk factors for the development of AMD have been described. The most commonly reported environmental risk factors include aging, smoking, family history, low dietary intake of antioxidants and omega-3 fatty acids, and reduced physical activity.[44, 192, 204] In addition, multiple genetic risk factors have been identified, consisting of genetic variants that are either common or rare in the population. A large risk effect has been reported for genetic variants located at the *CFH* and *ARMS2*/*HTRA1* loci.[101] Most genes associated with AMD can be clustered into 5 main pathways: the complement pathway, lipid transport, extracellular matrix

Table 1. Explanation of terms used in this review to describe different types of AMD

Type of AMD	Criteria
Any AMD	No specific definition of AMD reported or analyses were performed on all AMD stages together Early AMD Analyses were performed on AMD cases in the absence of advanced stage disease (GA or CNV) and can include early and/or intermediate AMD
Advanced: GA	Geographic atrophy of the RPE secondary to AMD
Advanced: neovascular	Choroidal neovascular lesion (active or occult) secondary to AMD, including serous and/or hemorrhagic RPE detachment, subretinal fibrovascular tissue and scarring
Any advanced AMD	No specific definition of advanced AMD reported or analyses were performed on both advanced AMD stages (GA and CNV) together
Dry AMD	No specific definition of dry AMD reported or analyses were performed on AMD cases in the absence of advanced neovascular AMD (can therefore include early AMD and/or advanced: GA)

AMD, age-related macular degeneration; CNV, choroidal neovascularization; GA, geographic atrophy; RPE, retinal pigment epithelium.

(ECM) remodeling, angiogenesis, and cell survival.[100] Despite considerable progress in understanding the genetic architecture of AMD, the exact disease etiology is not yet fully understood.

In attempts to unravel the etiology of AMD, to improve patient stratification, to monitor disease progression, and to discover new drug targets, many biomarker studies have been performed. In general, new analytical strategies have emerged, moving from single markers toward complex biomarker signatures, increasing the chance for greater specificity and a higher diagnostic or predictive value.

There has been no comprehensive overview of all potential biomarkers and their applicability in AMD. Here, we present a detailed summary of the current literature on molecular compounds reported as analyzed in serum, plasma, aqueous humor, vitreous, and urine of AMD patients. The scope of this review is limited to nongenetic chemical compounds. For all compounds, a short description of their function is provided, and the results of the studies are summarized and discussed in relation to AMD. Currently, most of these markers are not yet established as routine clinical diagnostic tools and are discussed here to direct future research and eventually clinical implementation.

2.2 NEOVASCULARIZATION AND HEMOSTASIS

Because choroidal neovascularization is one of the subtypes of advanced AMD, it is not surprising that the factors involved in neovascularization and hemostasis have been extensively studied. The results of the studies describing these factors are described in Sections 2.2.1, 2.2.2, respectively. A complete overview of the studies and references is provided in Supplementary Table 1.

2.2.1 Neovascularization

2.2.1.1 Vascular endothelial growth factor and soluble VEGF receptor 1

Vascular endothelial growth factor (VEGF) is currently the most important target in the treatment of nAMD, and the expression profile of VEGF has been extensively investigated in AMD patients. VEGF acts as a hypoxia-driven local signal to induce the formation of new blood vessels. Treatments inhibiting its function can partially restore and/or maintain vision in nAMD patients.

Contrary to expectation, VEGF is not consistently upregulated in AMD patients across studies. One study showed that VEGF levels in the aqueous humor of 12 nAMD patients were highly elevated (668.9 pg/mL) compared with 10 controls (cataract patients; 108.3 pg/mL).[334] In a second study involving aqueous humor, however, significant higher VEGF levels could only be demonstrated in the most aggressive form of nAMD (type 3 neovascularization) compared with controls.[74] A third study did not report a difference in VEGF levels in aqueous humor between nAMD and controls at all.[290] Of note, a considerable range in VEGF levels in aqueous humor exists among these studies. In the study by Tong and colleagues,[334] the levels of VEGF in control individuals were around 100 pg/mL, whereas the VEGF levels in the 2 other studies were much lower in controls (39.5 pg/mL and 63.9 pg/mL, respectively).[74, 290] These differences may be explained by the use of 3 different analytical systems, emphasizing the need for standardized assay systems for key marker compounds in eye fluids. In addition, studies analyzing VEGF levels in vitreous samples did not detect differences between VEGF levels of nAMD cases and controls.[135, 142]

Although the measurement of VEGF levels in vitreous or aqueous humor is expected to best reflect VEGF levels in the macula, the procedure is invasive and therefore not desirable in individuals with early or intermediate AMD. Thus, for purposes of a clinical tool for diagnosis and progression, measurement of VEGF levels in more

accessible body fluids such as serum or plasma is preferable. Several studies did investigate VEGF levels in AMD patients and controls in serum or plasma, with mixed results. Four studies detected significantly upregulated levels of VEGF in serum or plasma,[6, 115, 205, 338] but these findings are contrasted with 10 other studies that reported no association.[41, 80, 112, 122, 125, 211, 231, 299, 353, 380]

VEGF signaling is mediated through a complex of receptors and coreceptors, of which the soluble form of VEGF receptor 1 has been investigated in a number of studies. As in the case of VEGF, these studies do not offer a clear direction of effect. One study investigated the levels of soluble form of VEGF receptor in vitreous and found that levels were higher in nAMD patients.[142] In contrast, 2 studies performed on serum could not corroborate these findings. One of the studies did not find any association,[256] the other even reported lower levels of soluble form of VEGF receptor in nAMD.[340]

2.2.1.2 Pigment epithelium–derived factor

Pigment epithelium–derived factor (PEDF) is produced by RPE cells and has antiangiogenic properties, opposing the effects of VEGF. It has been proposed as a target to inhibit choroidal neovascularization and its expression signature in model systems suggests that it is downregulated under hypoxic conditions.[139] Two studies on vitreous support this by demonstrating a marked reduction in PEDF levels in AMD patients versus controls.[135,142] One study analyzing aqueous humor showed the opposite result, an increase of PEDF levels in AMD patients.[334] These conflicting results are not readily explained. It is possible that in different fluids or in different parts of the eye (anterior/posterior), PEDF is regulated differently, but additional experiments are needed to determine the direction of the effect with certainty.

2.2.1.3 Transforming growth factor beta

Transforming growth factor beta (TGF-β) has been described to increase the expression of VEGF and is therefore also implicated in neovascularization.[20] In vitreous samples of nAMD patients, TGF-β was significantly elevated when compared with controls (patients with idiopathic macular holes).[20] An earlier study had already demonstrated that urinary TGF-β levels were increased in cases compared with controls, but only in early AMD was the difference significant.[121]

2.2.2. Hemostatic system

2.2.2.1 Fibrinogen

Fibrinogen is a hemorheological factor involved in endothelial functioning.[34] Abnormalities in this factor are linked to thrombogenesis and vascular disorders[166]; hence, fibrinogen has been examined for its potential involvement in AMD. Studies have yielded mixed results. A number showed that increased fibrinogen level is a significant risk factor,[65, 205, 223, 271, 321] whereas others did not find evidence for an association.[58, 149, 185, 188, 288, 295, 331, 352,367]

2.2.2.2 Plasminogen activator inhibitor 1

Plasminogen activator inhibitor 1 is another main component of the fibrinolytic system.[29] Four studies have investigated whether a relation between plasminogen activator inhibitor 1 and AMD exists, with some support for a positive association,[367] whereas other studies did not find any association.[26, 288, 352]

2.2.2.3 Platelet count

Several studies have measured platelet count. Most did not find any association between platelet counts and AMD.[149, 180, 181, 186, 205] Two larger studies did find lower platelet counts in AMD, but this minimally protective effect for platelets on the development of AMD was only significant in univariate analyses.[50, 285]

2.2.2.4 Von Willebrand factor

Von Willebrand factor is a blood glycoprotein that is essential for normal hemostasis.[289] Because vascular pathology is hypothesized to be involved in the pathogenesis of AMD, Von Willebrand factor was investigated as a possible risk factor. One study showed higher levels compared with controls (but in multivariate analysis no significant correlation was found),[205] and 3 more studies found no association at all.[288, 352, 367]

In summary, many inconsistent results for factors involved in neovascularization have been reported, and further work is required to determine whether these could be used as AMD biomarkers. Factors involved in hemostasis described in Section 2.2.2 are unlikely to serve as biomarkers for AMD.

2.3 OXIDATIVE STRESS

The human body is dependent on an aerobic environment for survival. This constant exposure to oxygen can lead to detrimental oxidative modifications of cell components and tissues. Usually, cells are equipped with sufficient antioxidative mechanisms to maintain oxidant homeostasis, but if this balance is disrupted, oxidative stress occurs.[38] Oxidative stress in cells and tissues is characterized by an excess in molecules containing free radicals such as reactive oxygen species (ROS) and reactive nitrogen species.

Polyunsaturated fatty acid (PUFA) molecules are present in lipids on the membranes of cells and are prone to oxidation due to the presence of susceptible double carbon bonds.[38, 245] During the process of lipid peroxidation by ROS, the double carbon bond is oxidized, leading to the formation of unstable reactive carbonyl compounds (e.g., malondialdehyde [MDA]).[18, 22, 263, 357] ROS can also oxidize proteins, resulting in 2-(ω-carboxyethyl) pyrrole (CEP) protein adducts[117] and induce formation of advanced glycosylation end products (e.g., N^{ε}-carboxymethyllysine).[146, 304]

Increased oxidative stress is thought to be one of the underlying factors in the occurrence of AMD.[18, 24, 38, 245,333, 374] The eye, and especially the macula, is susceptible to oxidative stress because of its high metabolic activity and high PUFA content in the membranes of the photoreceptors.[38] High oxygen pressure from the blood in the choroid and exposure to bright light also causes increased ROS levels in the retina.[22, 263, 333] In addition, photoreceptors are subjected to constant shedding, and subsequent phagocytosis of the shed fragments leads to ROS generation.[38, 374] Environmental factors such as smoking and alcohol consumption can also increase ROS production.[346] Therefore, factors related to oxidative stress could potentially be valuable biomarkers for the incidence and/or progression of AMD and are discussed in more detail in Sections 2.3.1, 2.3.2, 2.3.3, 2.3.4. A schematic overview of these oxidative stress related factors is provided in Figure 1 and a complete overview of the studies and references is provided in Supplementary Table 2.

2.3.1 Oxidation products

2.3.1.1 Malondialdehyde

MDA is one of the reactive carbonyl compounds originating from PUFA oxidation, and its presence is often used to measure lipid peroxidation levels in blood or serum

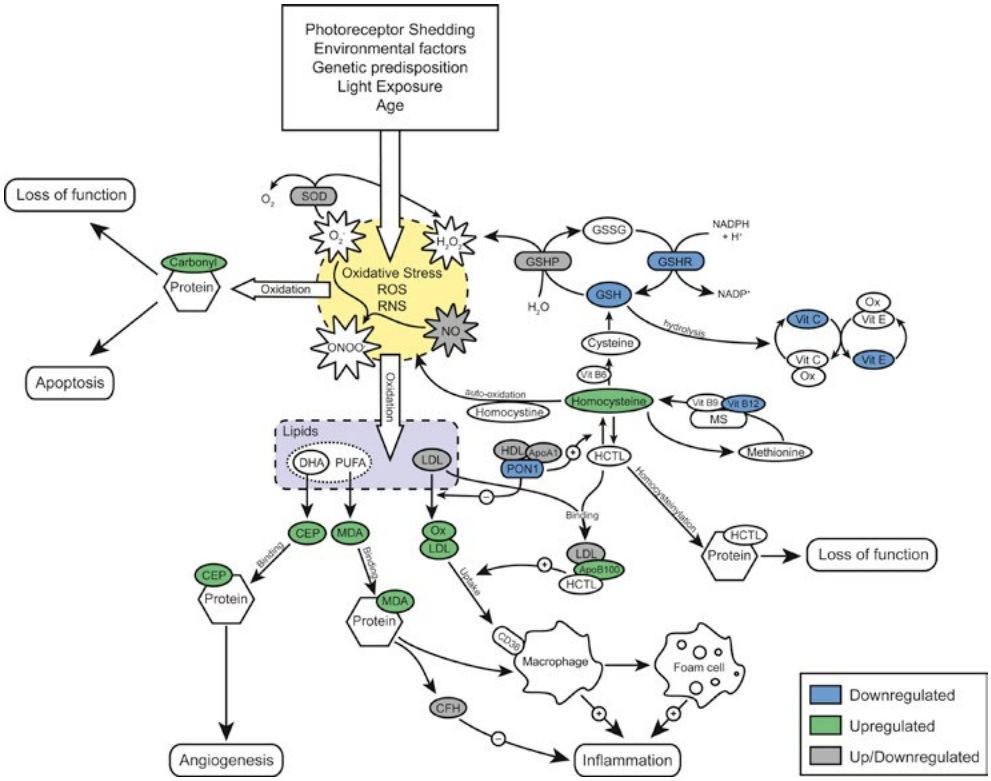

Figure 1. Networks of oxidative stress in age related macular degeneration (AMD). Spheres are colored to indicate levels in AMD patients compared to controls based on literature: upregulated (green), downregulated (blue), or inconsistent levels (gray). In this figure, studies reporting no association were not taken into account for the sake of readability. Apo, apolipoprotein; CEP, 2-(ω-carboxyethyl) carboxyethylpyrrole; DHA, docosahexaenoic acid; GSH, glutathione; GSHP, glutathione peroxidase; GSHR, glutathione reductase; HCTL, homocysteine thiolactone; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MDA, malondialdehyde; MS, methionine synthase; Ox, oxidized; PON1, paraoxonase 1; PUFA, polyunsaturated fatty acid; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; Vit, vitamin.

samples.[18, 22, 335] Increased systemic levels of MDA have been consistently observed in both wet and dry AMD.[18, 22, 86, 155, 263, 311, 335, 336, 346, 374, 375] In addition, an allele-dependent increase of MDA levels was measured in subjects carrying the A69S variant (rs10490924) in the *ARMS2* gene that is associated with AMD. Patients heterozygous or homozygous for the risk allele showed higher MDA levels.[263]

MDA is a highly reactive molecule that forms covalent bonds with the amino acids of endogenous proteins. This MDA modification can be recognized by factors of

the innate immune system such as complement factor H (FH), immunoglobulin M (IgM), and macrophages.[53, 357, 358] Binding of MDA by IgM or macrophages leads to a proinflammatory response by increasing the expression of the inflammation factor interleukin (IL)-8,[274, 358] whereas binding to FH attenuates inflammation.[358]

2.3.1.2 CEP adducts and N(6)-carboxymethyllysine (CML)

Docosahexaenoic acid (DHA) accounts for about 80% of all PUFAs in the photoreceptor outer segments and is most prone to oxidation in human tissues.[96] Upon oxidative stress, DHA is oxidized forming specific CEP adducts.[117] Plasma CEP levels in AMD patients are elevated compared with controls.[118, 242, 351] Moreover, elevated CEP levels combined with AMD risk alleles in *ARMS2*, *HTRA1*, *CFH*, or *C3* increased the risk of AMD twofold to threefold compared with genotype alone.[118]

Furthermore, plasma of AMD patients contained more and a higher diversity of CEP autoantibodies compared with controls in 2 studies from the same group.[117, 118] Another independent study found no association between CEP autoantibodies and AMD.[242]

CML is an advanced glycation end product that originates from a protein lysine modification and is a major immunological epitope recognized by the immune system.[146] Plasma CML levels were upregulated in AMD in one study,[242] but no significant difference was found in another.[304]

Both CEP adducts and CML are present on proteins. They are recognized by the immune system[146, 359] and can stimulate angiogenesis in vivo.[78, 248] Receptor-mediated binding of CEP adducts results in an angiogenic response of endothelial cells independent of VEGF signaling.[359] Upregulation of CML and CEP levels in AMD might be implicated in the progression toward nAMD by promoting angiogenesis, but further studies are necessary to support this hypothesis.

2.3.1.3 Protein carbonyl groups and total oxidation status

Levels of protein carbonyl groups are often used to assess the total protein oxidation status in subjects as they are easy to measure.[64] Protein carbonyl groups consist of an oxygen molecule bound to a carbon atom with a double bond (-RC=O) resulting from protein oxidation and are therefore indicative of oxidative stress. Elevated levels of both protein carbonyl group[336, 379] and total oxidation status[336, 341] were found in nAMD patients.

2.3.1.4 Oxidized low density lipoprotein

Low-density lipoprotein (LDL) is abundantly present in and around cells and is an easy target for oxidation by ROS. LDL cholesterol (LDL-C) has been studied extensively in the context of AMD, described in Section 2.5.2; however, studies on its oxidized form (oxidized low density lipoprotein [Ox-LDL]) are more limited. Higher Ox-LDL levels were found systemically in AMD patients compared with controls,[147, 152, 153] but a lack of association has also been reported.[184]

Increased Ox-LDL levels are known to activate various factors of the complement system such as C3b, C5b-9, and complement factor B (FB).[79] These factors are described in more detail in Section 2.4.1. High Ox-LDL levels as observed in AMD might initiate apoptosis of RPE cells through disruption of the mitochondrial pro- (Bax) and antiapoptotic (Bcl2) balance,[373] leading to GA. In addition, uptake of Ox-LDL molecules by macrophages contributes to the formation of foam cells, implicated in the development of atherosclerotic plaques.[270]

2.3.2 Nitric oxide

Nitric oxide is one of the most abundant free radicals in the human body and is able to react with other ROS resulting in cell dysfunction and apoptosis.[86] It is synthesized by endothelial cells and is an important vasoactive agent affecting blood flow and other vascular functions.[28] Involvement of nitric oxide in AMD is less clear. One study observed increased levels of nitric oxide in AMD patients,[86] another study described downregulation of nitric oxide in nAMD,[335] and a third study reported no association.[338]

2.3.3 Homocysteine

Homocysteine is an intermediate molecule in the conversion of the amino acid methionine to cysteine and glutathione (GSH), a process mediated by multiple enzymes.[104, 294] Homocysteine can autooxidize in plasma, leading to the formation of various reactive products such as homocysteine thiolactone, which is also accompanied by ROS generation (Figure 1).[60]

Dysregulation of the homocysteine balance has been associated with various diseases such as vascular dysfunction, autoimmune diseases, and neurodegenerative disorders.[294] Increased systemic levels of homocysteine were observed in both neovascular and dry forms of AMD compared with controls,[18, 19, 60, 110, 113,152, 153, 168, 213, 284, 300, 347] and there were also higher levels in the

vitreous of nAMD patients.[213] Moreover, some studies found higher homocysteine levels in nAMD compared with dry AMD[19, 110]; however, other studies did not find an association between homocysteine levels and AMD.[54, 132, 188, 247, 352, 367]

2.3.4 Antioxidants

Antioxidants enhance ROS clearance and prevent ROS formation thereby averting damage in the aging eye and other tissues.[333] Enzymes such as catalase, superoxide dismutase, and paraoxonase prevent the accumulation of oxidized lipids by converting ROS before they can react or by removing the oxidized products from the endogenous proteins.[333] Several vitamins and trace elements act as cofactors for these enzymes, or react with ROS to prevent accumulation.[357, 374]

Multiple studies hypothesized that the antioxidant capacity in AMD patients might be impaired, and some showed a decreased overall antioxidant capacity in serum of patients.[58, 87, 269, 311, 336, 379] In the following sections, we discuss levels of thiols (Section 2.3.4.1), carotenoids (Section 2.3.4.2), and enzymes with antioxidant activity (Section 2.3.4.3) in AMD patients.

2.3.4.1 Thiols and GSH

Thiols mediate an important part of the balance between proper oxidation versus antioxidants in tissues. Their main characteristic is a carbon-bonded sulfhydryl group (C-SH), which can form a disulfide bridge with other thiols via redox reactions (C-S-S-C). Thiols can neutralize ROS by providing an electron during the formation of the disulfide bridge.[218] Although their normal function is to prevent oxidative stress, thiols can also promote oxidative stress in the presence of metal ions such as iron.[152]

Thiol content is either measured by focusing on the individual thiols or by evaluating total thiol (tSH) content of the blood. GSH is one of the most important thiols in the body. GSH can be transformed into glutathione disulfide (GS-SG) by the enzyme glutathione peroxidase (GSHP), thereby breaking down hydrogen peroxide (2 GSH + H_2O_2 → GS-SG + 2 H_2O).[218] Glutathione reductase (GSHR) is able to transform the formed glutathione disulfide to its monomeric form (GS-SG + NADPH + H^+ → 2 GSH + $NADP^+$), making it available for conversion by GSHP again.[218] This circular process (Figure 1) is of vital importance for proper ROS maintenance.

Lower levels of GSH and tSH are thought to result in more ROS formation owing to the absence of hydrogen peroxide clearance, resulting in subsequent oxidative

damage.[60, 333] Lower levels of total thiol content[60, 152, 341]and plasma GSH[60, 152] were found in patients with AMD compared with control subjects, and both were negatively correlated with homocysteine levels[60]; however, multiple studies have found no association between systemic GSH levels and AMD.[35, 72, 273, 291, 375]

Plasma and serum GSHR levels were lowered in patients with AMD,[55, 58, 379] although 1 study did not find this association in erythrocytes.[68] Systemic GSHP levels were lowered in some studies[85, 269, 272, 346] and higher in 1 study,[71] but in most studies, no association was found.[55, 58, 68, 375, 379]

2.3.4.2 Carotenoids

Carotenoids are a group of natural red and yellow hued pigments (carotenes and xanthophylls) synthesized in most plants. The antioxidant capacity of carotenoids is based on their ability to absorb and process free electrons from ROS such as singlet oxygen ($^1O_2$) and peroxyl radicals ($ROO^{\bullet}$). After the uptake of an electron, the carotenoid releases its energy in the form of heat and can be used again. Humans are unable to synthesize carotenoids and rely on dietary intake of vegetables.[95, 325] In AMD, total serum carotenoid levels were decreased in 2 studies by the same group,[87, 88] whereas 2 other studies described a lack of association.[40, 313]

Two main xanthophylls are located in the macula: lutein is concentrated in the peripheral macula and zeaxanthin in the fovea. Here, they are able to attenuate blue light wavelengths, preventing the light from reaching and damaging the underlying photoreceptors.[195] In blood, lutein and zeaxanthin are transported by lipoproteins such as high-density lipopotein (HDL) and LDL. Zeaxanthin and lutein exert their antioxidant abilities by reacting with free radicals both in the macula and in blood.[195] Levels of lutein and zeaxanthin were found to be decreased in AMD patients in several studies.[70, 87, 384] One study described decreased levels of zeaxanthin but not lutein in AMD patients.[103] Others found no association for either lutein or zeaxanthin.[40, 214, 224, 292, 313]

β-cryptoxanthin is a carotenoid most commonly found in citrus fruits. Besides its role as an antioxidant, in vitro experiments have shown that β-cryptoxanthin also stimulates DNA repair mechanisms.[206] Levels of β-cryptoxanthin were decreased in patients with advanced AMD in some studies,[87, 224, 313, 384] whereas others did not find a

significant association with AMD.[40, 70, 214, 292]

A decrease of α-carotene was found in patients with nAMD,[87, 384] whereas higher levels of α-carotene were present in early AMD.[384] Also β-carotene levels were decreased in advanced AMD in some studies[87, 224, 384]; however, most studies did not find a significant association between AMD and α-carotene or β-carotene levels.[40,70, 214, 224, 292, 313, 322, 360] Importantly, supplementation of β-carotene has been associated with an increased risk of lung cancer in smokers and former smokers, and therefore, long-term use to inhibit AMD progression is not recommended.[5, 251]

Finally, one of the most potent antioxidants present in blood is lycopene. The main dietary sources of this red pigment carotenoid are red fruits or vegetables, such as tomatoes.[102] Levels of lycopene were either decreased in AMD patients[40, 313, 384] or not associated with AMD.[70, 87, 214, 224, 292]

In summary, when studies reported a significant association between carotenoids and AMD, the vast majority described decreased carotenoid levels in patients. This probably reflects a difference in dietary intake of these carotenoids between AMD patients and controls. Several studies reported that a higher intake of carotenoids is associated with a reduced risk of AMD.[332, 344, 365] In addition, a beneficial effect was shown for supplementation with lutein and zeaxanthin on progression to advanced AMD.[3, 4, 5]

2.3.4.3 Enzymes

2.3.4.3.1 Superoxide dismutase

Superoxide dismutase (SOD) is an important antioxidant that catalyzes the conversion of superoxide ($O_2^{\bullet -}$) into oxygen and hydrogen peroxide (H_2O_2).[333] Two families of SOD exist based on their metal ion cofactor: SOD1 (CuZnSOD), which is localized to the cytoplasm and SOD2 (MnSOD), found in mitochondria.[333] The absence of SOD1 or SOD2 has been associated with early retinal cell degeneration in mice,[129, 164] suggesting an important role for SOD in the eye.

With regard to AMD, several reports show elevated systemic SOD activity in AMD patients compared with controls,[10, 155, 310, 311] others found lowered SOD activity levels,[86, 272, 346, 375, 379] and still others measured no significant association.[55, 58, 68, 71, 269] One study showed a significant difference in SOD activity between late and early AMD, with a lower SOD activity in late AMD patients.[86]

The association of both low and high SOD serum activity levels with AMD might be explained by the damaging effects of both high and low levels of SOD. High levels of SOD lead to higher H_2O_2 production, whereas low SOD activity leads to the continuing presence of $O_2^{\bullet-}$ molecules. The detrimental effects of both low SOD and high SOD activity on ROS production suggest that imbalance of the enzyme activity leads to pathological conditions and that proper SOD balance is important to maintain homeostasis.

2.3.4.3.2 Paraoxonase 1

Paraoxonase 1 (PON1) is bound to HDL. PON1 hydrolyzes organophosphates and lipid peroxides and inhibits the oxidation of LDL.[18, 153] In addition, PON1 is able to detoxify homocysteine thiolactone, one of the highly reactive metabolites of homocysteine.[151] Active PON1 interacts with oxidized proteins or lipids, leading to its own inactivation.[17] The low serum PON1 activity levels observed in AMD patients[18, 22, 341] could be due to inactivation of PON1 after reacting with oxidized proteins.

2.3.4.3.3 Catalase

Catalases are important in ROS clearance by converting hydrogen peroxide (H_2O_2) to oxygen and water.[47] In AMD, 3 studies reported downregulated systemic catalase activity levels,[272, 346, 374] whereas 3 others reported no difference in catalase activity levels between AMD patients and controls.[68, 86, 269]

Taken together, dysregulation of the oxidative stress pathway and the manner in which oxidative stress is managed by the body seems to play an important role in AMD. A large number of investigators have reported decreased levels of antioxidants and elevated oxidized protein or lipid levels (Figure 1). The most promising biomarker candidates in the oxidative stress pathway are MDA and homocysteine, which were consistently reported to be increased in AMD patients. For other factors, however, the reported associations were less clear and with mixed results. This could indicate that an imbalance of the entire oxidative stress system may play a role, rather than levels of individual factors of this system specifically.

2.4 IMMUNITY

The involvement of the immune system in the pathology of AMD is widely accepted, and some suggest reframing AMD as an autoimmune disease.[39] The activity of the immune system in AMD, both innate and adaptive, has been implicated at several levels. Immune cell infiltrates have been shown in the retinas of AMD patients examined postmortem,[198] with evidence of cytokine/chemokine expression at the affected site, as described in more detail in Section 2.4.2.

Strong evidence for the involvement of the immune system in AMD also comes from several GWAS studies (described in Section 2.1).[99, 101] In particular, the role of the complement system is apparent. In the following sections, we discuss immunity-related compounds, including systemic markers of the complement system (Section 2.4.1) and elements of adaptive and innate immunity (Sections 2.2, 2.3, 2.4). A complete overview of the studies and references is provided in Supplementary Table 3.

2.4.1 The complement system

The complement system is an integral part of innate immunity with essential roles in protection against foreign intruders via tissue inflammation, cell opsonization, and cytolysis. It is also involved in monitoring and maintaining host tissues by clearing cellular debris, maintaining cellular integrity, tissue homeostasis, and modifying the adaptive immune responses.[105]

Ever since histopathological studies demonstrated the presence of complement components in drusen,[11, 127] the involvement of the complement system in AMD has been studied extensively and genetic evidence showing strong links between components of the alternative pathway of the complement system and AMD followed.[101,193] Although the complement system acts locally, its components can also be detected systemically in serum or plasma. A number of studies have investigated the expression levels of complement regulators, complement components, and activation products in AMD patients versus controls. An overview of the alternative pathway of the complement system is provided in Figure 2.

The central molecule of the complement system is complement component 3 (C3). Enzymatic cleavage of C3 results in the generation of its active fragments C3a (a potent proinflammatory molecule) and C3b that, via several digestion steps, leads to C3d.[220]

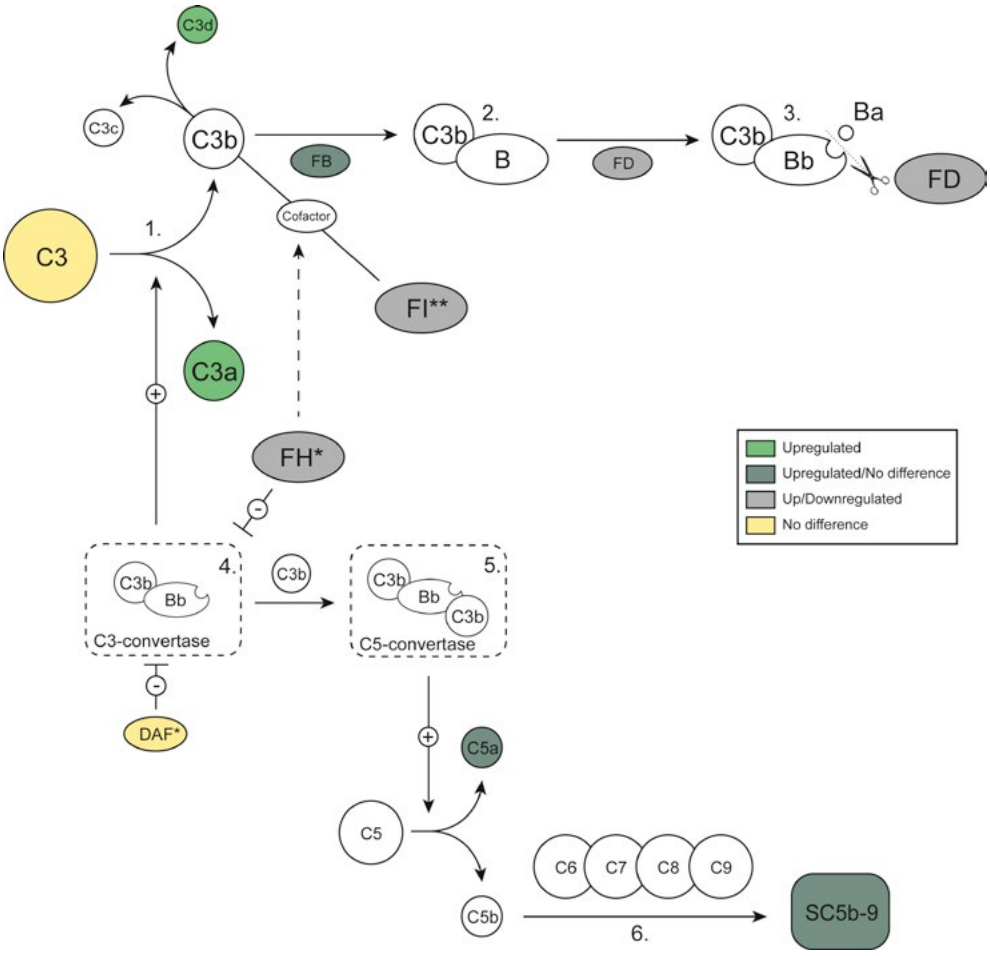

Figure 2. Overview of the alternative pathway of the complement system. Spheres are colored to indicate levels in AMD patients compared with controls based on literature: upregulated (green), upregulated/no difference (dark green), upregulated/downregulated (gray), and no difference (yellow). (1) Complement component 3 (C3) splits into C3a and C3b by spontaneous hydrolyzation or by the C3-convertase (C4bC2) resulting from activation of the classical or lection pathway. (2) Factor B (FB) can bind C3b to form C3bB. (3) The bound factor B is then cleaved by factor D (FD) which results in the formation of the C3-convertase: C3bBb (4). This C3-convertase can cleave C3 which leads to more C3b and in turn increased formation of the C3-convertase (known as the C3 amplification loop). The C3-convertase can also bind another C3b molecule to form C3bBb3b, which is a C5-convertase (5). This C5-convertase can convert C5 into C5a and C5b. (6) C5b then sequentially binds C6, C7, C8, and multiple C9 molecules to form the terminal complement complex (SC5b-9), also known as membrane attack complex. * The C3-convertase is inhibited by several complement regulators, among which decay accelerating factor (DAF) and factor H (FH). ** Factor I (FI) can breakdown C3b via several digestion steps to C3c and finally C3d, this protease activity, however, requires a cofactor, such as FH.

A number of studies measured systemic C3 levels but did not find an association with AMD,[278, 298, 312, 319] whereas higher systemic levels of its active fragments, C3a and C3d, were detected in AMD patients.[130, 278, 298, 319] These findings suggest that the processing of C3, that is its activation, may be associated with AMD and a number of studies have investigated this. Complement activation was measured as the ratio of C3 and its degradation product C3d (C3d/C3)[280, 281, 319] or as a cleaved form of C3a (C3a-desArg) in blood[317] and urine.[121] Of the 5 studies that investigated complement activation in AMD, 4 found higher complement activation levels in AMD patients.[280, 281, 317, 319] An association of C3a-desArg in urine with AMD was not established.[121] A recent study suggests that complement activation levels may decrease at more advanced stages of the disease, but this finding needs to be confirmed in prospective AMD cohorts.[320]

Besides C3, complement component 5 (C5) is also essential in the activation cascade because it serves as the entry point for the formation of the terminal complement complex (SC5b-9).[220] The activation product of C5, C5a, is a potent anaphylatoxin. Increased levels of C5a were detected in most, but not all[130] studies examining the role of C5a in AMD.[278, 298, 319] These same studies also tested whether SC5b-9 is associated with AMD. Higher SC5b-9 levels were detected in AMD in 1 study,[298] but the other 2 studies found no evidence for an association.[278, 319]

The activity of the complement system is tightly controlled by regulatory factors that ensure appropriate, but not excessive, generation of terminal complexes. Among others, they include complement FH (encoded by the *CFH* gene), factor I (FI, encoded by *CFI*), FB (encoded by *CFB*), factor D (FD, encoded by *CFD*), and decay accelerating factor (DAF/CD55, encoded by *CD55*).[220]

Genetic association studies showed strong evidence of an association between the *CFH* gene and AMD.[101] Systemic levels of FH have been investigated with mixed results, however. Four studies report lower FH levels in AMD,[14, 278, 308, 310] 1 study detected higher levels of FH in AMD,[128] and another 4 studies did not find an association with AMD.[120, 298, 312, 319]

Similar to FH, FI also inhibits the activity of the complement system through inactivation of C3b. Genetic evidence for FI involvement in AMD has been shown previously, but no conclusive evidence links FI levels to AMD in general. One study reports increased FI levels in AMD,[312] another reports decreased levels but only in patients carrying a rare genetic variant in *CFI*,[343] and 2 did not find any association.[278, 319]

The findings for FB and FD levels in AMD are also inconsistent. Three studies reported higher FB levels in AMD patients,[130, 298, 319] whereas 2 others did not detect an association with AMD.[278, 312] Similar results were described for FD, where 3 studies reported higher FD levels,[130, 298, 326] 1 study reported lower levels in AMD,[312] and another found no association with AMD.[278] Finally, 2 studies that examined the role of CD55 did not find evidence for an association with AMD.[123, 314]

In summary, not only genetic studies but also studies measuring complement components provide evidence that link complement activation to AMD (Table 2). Some factors, however, should be taken into account when considering the use of systemic complement activation levels as a biomarker for AMD in individual patients. Often antibody-based tests do not discriminate between the total amount of a specific complement factor and its processed activated part, as cleavage of the preform to the active mature form cannot be distinguished by the reagent. Moreover, complement activation levels are subject to high variability, and other causes of increased complement activity should be excluded because increased complement activation may reflect immune system activity that is not necessarily connected to disease progression. Linking exacerbated complement activation in an individual patient to his or her genetic blueprint is potentially more useful. For example, haplotypes and combinations of genotypes in several complement genes have been associated with increased complement activation levels.[130, 266] In addition, several investigations have now demonstrated that FI levels are lower in AMD patients carrying rare genetic variants in the *CFI* gene.[107, 172, 343] For FH levels, there were similar associations with genotype. Some but not all rare variants in the *CFH* gene were associated with reduced FH levels.[337, 349, 378] Thus, patients carrying rare variants in complement genes tend to have higher complement activation levels than AMD patients in general.[106] These insights may benefit ongoing clinical trials on the effectiveness of complement inhibitors and could prioritize patients who carry rare variants in these genes.

2.4.2 Cytokines

2.4.2.1 Interleukins

Cytokines are a large family of small proteins that play a pivotal role in cell signaling. An important group of cytokines are interleukins. Interleukins play a key signaling role in the inflammatory response. Interleukin-6 (IL-6) is a cytokine with many described functions,[215, 225] and its relationship to AMD has been investigated. A number of studies reported increased levels of IL-6 in AMD patients,[7, 124, 191] but the majority found no

Table 2. Overview of studies measuring complement components in AMD patients compared with controls

Component	Upregulation	No difference	Downregulation
C3		Scholl et al, 2008298 Reynolds et al, 2009278 Silva et al, 2012312 Smailhodzic et al, 2012319	
C3a	Scholl et al, 2008298 Reynolds et al, 2009278		
C3d	Scholl et al, 2008298 Hecker et al, 2010128 Smailhodzic et al, 2012319		
C3a des Arg C3d/C3	Sivaprasad et al, 2007317	Guymer et al, 2011121	
C3d/C3	Smailhodzic et al, 2012319 Ristau et al, 2014280 Ristau et al, 2014281		
C5a	Scholl et al, 2008298 Reynolds et al, 2009278 Smailhodzic et al, 2012319	Hecker et al, 2010130	
SC5b-9	Scholl et al, 2008298	Reynolds et al, 2009278 Smailhodzic et al, 2012319	
FH	Hakobyan et al, 2008128	Scholl et al, 2008298 Silva et al, 2012312 Smailhodzic et al, 2012319 Guymer et al, 2015120	Reynolds et al, 2009278 Ansari et al, 201314 Sharma et al, 2013308 Sharma et al, 2013310
FI	Silva et al, 2012312	Reynolds et al, 2009278 Smailhodzic et al, 2012319 Van de Ven et al, 2013a,343	
FB	Scholl et al, 2008298 Hecker et al, 2010130 Smailhodzic et al, 2012319	Reynolds et al, 2009278 Silva et al, 2012312	
FD	Scholl et al, 2008298 Hecker et al, 2010130 Stanton et al, 2011326	Reynolds et al, 2009278	Silva and colleagues 2012312
DAF/CD55		Haas et al, 2011123 Singh et al, 2012314	

a Significant downregulation of FI was described in a subgroup of patients with a rare variant in the CFI gene.

association with AMD in general.[15, 58, 183, 188, 231, 240, 290, 352, 367] Notably, a number of these studies did find an association in subgroup analyses. For instance, an association with AMD was reported only in patients with high IL-6 levels[58] or the association with IL-6 was established only for GA patients.[188] In addition, only the highest tertile of IL-6 levels was associated with progression of AMD in a prospective cohort study.[303]

Other interleukins have also been studied in relation to AMD, although to a lesser extent. In most studies, these interleukins were measured in a multiplex analysis of inflammatory markers. Two studies measured multiple interleukins in serum.[231, 240] In 1 study, there were higher serum levels of IL-1β, IL-4, IL-5, IL-10, and IL-13 in patients with nAMD,[240] but these factors were not associated with early, atrophic, or neovascular AMD in another study.[231] Higher serum levels of IL-1α and IL-17 in nAMD patients were only reported in the first study. In addition, no association was found for IL-2, IL-12, and IL-15.[240] Other studies also detected no association between IL-2,[188] IL-15,[89] and AMD. For IL-8, although no association was present in 2 studies,[229, 235] a third larger study described higher IL-8 levels in AMD patients, in particular in dry AMD.[7] Higher IL-18 levels were reported in dry, but not nAMD, in 1 study.[143] A second study did not find different levels between different types of AMD and controls.[89]

Although most studies focused on systemic levels of interleukins, a small number performed measurements in aqueous humor[290] and vitreous.[383] Higher IL-1β levels were found in the vitreous of nAMD patients.[383] In aqueous humor, IL-1α and IL-15 were upregulated and IL-13 was downregulated, whereas for IL-2, IL-4, IL-8, IL-10, IL-12, and IL-17, no differences were detected.[290]

2.4.2.2 Chemokines and chemokine receptors

Chemokines (chemotactic cytokines) have the ability to direct movement of cells through receptor-mediated chemotaxis. Evidence from postmortem material and animal models have implicated infiltrating immune cells in pathological eye tissues, suggestive of the involvement of chemokines in these environments.[200, 287, 305, 329]

Chemokine ligand 2 (CCL2; or monocyte chemoattractant protein 1) attracts C-C chemokine receptor type 2 (CCR2)-expressing monocytes into tissues and is one of the most studied chemokines in AMD. Five relatively small, case-control studies did not find an association between levels of CCL2 and AMD,[92, 116, 120, 231, 290] but several larger studies did see an association with increased levels of CCL2.[9, 310, 382] This effect was also reported in a cross-sectional study linking higher levels of urinary CCL2

to early AMD.[121] Overall, these findings support the notion that CCL2 is involved in AMD. Interestingly, CCR2-expressing cells can also be detected systemically, and both decreased and increased levels have been associated with AMD.[9, 115] Two other studies did not find any association.[94, 376]

Another receptor involved in the recruitment of monocytes, CX3C receptor 1, was measured in two AMD studies.[92, 116] Only the more recent study reported CX3C receptor 1 to be upregulated in both early and neovascular AMD.[92]

Eotaxin (eosinophil chemotactic protein/CCL11) and closely related eotaxin-2 (CCL24) attract eosinophils. These are interesting molecules for AMD pathogenesis because CCL11 and CCL24 and their receptor CCR3 are implicated in choroidal neovascularization.[91, 309] CCR3 is expressed on choroidal neovascular endothelial cells and signaling through this receptor leads to endothelial proliferation, even without the involvement of eosinophils or other immune cells. Blocking CCR3 signaling in animals led to a potent inhibition of neovascularization, even stronger than blocking VEGFA signaling.[329] Levels of CCL11 were investigated in 2 studies, one reporting increased levels in AMD,[231] and the other finding no differences.[91] Supportive of the aforementioned findings, 2 studies of the same group reported CCL24 to be upregulated in AMD.[309, 310] Despite these overall promising results, systemic elevations of CCR3 on immune cells have not yet been reported. The only study investigating CCR3 on granulocytes reported no association, although there was a trend toward higher expression of CCR3 in nAMD.[91] Taken together, the CCL11/CCL24-CCR3 axis is potentially involved in human AMD pathology, but it is not yet clear whether this is mostly a local signaling, mediated through CCR3 expression on endothelial cells, or whether systemic CCR3-expressing cells could also be involved.

The chemokine ligand CXCL10, also known as interferon gamma-induced protein 10, attracts a range of cell types and is an inhibitor of angiogenesis.[13] Two studies showed no association with CXCL10 in serum or plasma and AMD,[93, 116] and only 1 study showed elevated serum CXCL10 levels in AMD patients.[231] Of interest is a recent publication, showing upregulation of CXCL10 in aqueous humor of AMD patients compared with controls undergoing cataract surgery,[290] suggesting that the effect of this chemokine might be local.

The receptor for CXCL10 is CXCR3 which is expressed on a variety of cell types. Only one study investigated numbers of CXCR3-expressing cells peripherally and detected

reduced presence of CD8+ T-cells expressing CXCR3 in AMD,[93] but additional research is warranted before concluding whether the CXCL10-CXCR3 axis can be reliably used as a biomarker for AMD.

It has been suggested that stem cell progenitor cells are involved in the disease etiology of AMD. Chemokine ligand CXCL12, also known as stromal cell-derived factor 1, plays a role in the movement of these stem cell progenitor cells throughout the body. Four small case-control studies have investigated the plasma levels of stromal cell-derived factor 1 in AMD patients with mixed results. Two studies, by the same group, report significantly lower levels of stromal cell-derived factor 1 in patients with nAMD,[210, 211] whereas another study showed the inverse effect,[299] and the fourth did not report any differences between nAMD and control individuals.[115]

2.4.2.3 Other cytokines

2.4.2.3.1 Tumor necrosis factor alpha

Tumor necrosis factor alpha, an important marker for systemic inflammation, has been investigated as such in several studies; however, no significant associations between AMD cases and controls were reported in serum or plasma.[120, 124, 183, 188, 231, 240, 380] Increased levels of soluble tumor necrosis factor alpha receptor 2 were reported in a case-control study in early and neovascular AMD,[89] which in a large population-based study did not reach statistical significance, but there was a trend toward upregulation in early AMD patients.[191]

2.4.2.3.2 Interferon gamma

Interferon gamma is an important cytokine in both innate and adaptive immunity as it induces cellular response to infections.[297] Three studies measured interferon gamma in AMD cases and controls, but none found an association with AMD.[89, 231, 240]

2.4.3 Other immune factors

2.4.3.1 C-reactive protein

C-reactive protein (CRP) is a marker of inflammation and a so-called acute phase protein because its levels change quickly upon disturbances of homeostasis. Evidence regarding the possible relation of this protein with AMD is inconclusive, with a roughly equal number of studies reporting higher CRP levels in AMD patients[7, 56, 57,138, 174, 228, 282, 290, 301, 302, 304, 342, 347, 356, 376] or no clear evidence for an association.[33, 58, 65, 120, 134, 141, 161,185, 217, 285, 312, 315, 327] Those that used a more precise

measurement of CRP (high-sensitivity CRP) were also not able to provide conclusive results: 5 studies detected higher levels of high-sensitivity CRP in AMD patients,[32,124, 191, 230, 295] compared with 5 that did not show an association with AMD.[15, 183, 188, 352, 367]

2.4.3.2 (Soluble) Intercellular adhesion molecule and vascular cell adhesion molecule

Intercellular adhesion molecule and vascular cell adhesion molecule are immunoglobulins that are usually upregulated on cell surfaces after immune signaling has taken place.[48] They form a sticky surface to which immune cells that express integrins can adhere. These molecules and their soluble counterparts are rarely investigated alone but usually as part of a panel that measures inflammatory activity. For intercellular adhesion molecule, 1 study reported higher levels to be associated with the incidence of AMD in women,[295] whereas 6 others did not find any association.[120, 134, 183, 191, 352, 367] In the case of vascular cell adhesion molecule, 1 study measured higher levels in AMD patients,[191] whereas 2 studies did not find any association with AMD.[120, 134] In addition, no association with AMD progression and either Intercellular adhesion molecule or vascular cell adhesion molecule was reported.[303]

2.4.3.3 White blood cell count

As mentioned previously in Section 2.4, a clear link with inflammation and inflammatory processes and AMD has been established, and several immune competent cells have been implicated in the disease etiology. As a result of local stress or inflammation, the body may respond by cellular proliferation of immune cells and recruitment of these cells to the affected site. From this perspective, white blood cell count is an interesting parameter to measure in AMD. A relatively large number of studies have investigated white blood cell count in AMD, and some did detect increased white blood cell numbers.[31, 181, 182, 191, 307, 356] This contrasts with most studies that did not find any association.[50, 113, 149, 157, 180, 181, 183, 185, 187, 205, 285, 315, 352, 367] Nevertheless, white blood cell count may still be considered as a potential biomarker for AMD if the analysis is performed in the context of a different theoretical framework. It is conceivable that it is not the total number of cells that change but rather the ratio between different cell types. Supporting this notion, a higher neutrophil/lymphocyte ratio has been associated to AMD and AMD subtypes.[148] A more in-depth analysis of the different cellular subtypes, such as the relative expression of cytokine/chemokine receptors,

would offer more insights.

2.4.3.4 Pentraxin-3

Pentraxin-3 (PTX3), like CRP, belongs to the pentraxin superfamily. Upon inflammation, PTX3 is produced locally by the RPE[162] and can interact with complement component C1q and enhances activation of the classical and lectin pathways of the complement system. In addition, PTX3 attracts complement FH, thereby inhibiting the amplification loop and preventing excessive activation of the alternative pathway.[76, 162] Although 1 case-control study reported higher plasma PTX3 levels in nAMD,[228] a more recent study (including also early AMD and GA patients) could not replicate these findings.[162] The latter study did however describe an increased expression of the *PTX3* gene with age- and inflammation-induced apical PTX3 secretion of the RPE.[162] Taken together, this suggests a more local expression of PTX3 in AMD; however, measurements of PTX3 locally in vitreous samples have not yet been performed and would therefore be a target of further research.

2.4.4 Antibodies

2.4.4.1 Antiretinal autoantibodies

The formation of antibodies against foreign epitopes is a key element of immunity. When endogenous epitopes become the trigger for mounting an immune response, autoimmunity ensues.[271] Antibodies against epitopes found in retinal material of AMD patients have been investigated in various studies. Several studies demonstrated upregulation of circulating antiretinal autoantibodies (ARAs) in the serum of AMD patients.[49, 119,264, 268] Although one study showed similar levels of ARAs in cases and controls, it did show a difference in types of antibodies specific for each disease stage.[2] In addition, higher concentrations of circulating ARAs were detected in treatment-naive nAMD patients compared with controls.[196, 197] These levels also correlated to lesion size.[197] After the loading phase of anti-VEGF treatment, autoantibody levels decreased.[196, 197] Moreover, correlations were reported between ARA levels and improvement of visual acuity, fluid reduction on optical coherence tomography, and decreased leakage on fluorescein angiography after 3 months.[197]

Furthermore, other studies attempted to identify specific circulating ARAs associated with AMD.[145, 156, 232] Surprisingly, one study showed not only upregulation of antibodies but also downregulation of a specific ARA in AMD. Lower antibody concentrations were reported for α-crystallin, whereas α-enolase and glial fibrillary acidic protein

antibodies were both significantly higher in serum of AMD patients.[156] The latter finding is supported by results from a previous study which showed different staining patterns in serum of AMD patients, with the most frequent pattern observed being almost identical to that using antiglial fibrillary acidic protein antibodies.[268] In addition, using an untargeted approach, 1 study identified 4 novel retinal antigens in serum of AMD patients: retinol binding protein 3 (Rbp3), aldolase C, pyruvate kinase isoform M2, and retinaldehyde binding protein 1.[232] Because Rbp3 and retinaldehyde binding protein 1 were previously reported in other ocular diseases, this study focused on aldolase C and pyruvate kinase isoform M2. A significant higher reactivity to aldolase C in nAMD, but not in early AMD, was reported. Because reactivity to pyruvate kinase isoform M2 was higher in both AMD groups compared with controls, this could potentially be a biomarker for the development of AMD.[232] A more recent study with a similar approach also identified ARAs with higher reactivity in AMD; heat shock 70 kDa protein 8 and 9, α-crystallin A chain, annexin A5, and protein S100-A9.[145]

2.4.4.2 Other autoantibodies

Serum autoantibodies have been extensively investigated by Morohoshi and colleagues using an antigen microarray analysis containing 85 autoantigens. Serum of AMD patients and controls showed a different IgG and IgM autoantibody profile, and multiple autoantibodies were significantly higher in AMD. In addition, they calculated IgG/IgM ratios for the antibodies and evaluated whether this ratio correlated to disease severity. Antiphosphatidylserine IgG/IgM was significantly elevated in AMD and correlated best with AMD stage. Moreover, reactivity to phosphatidylserine was highly increased in retina of AMD patients compared with controls.[232]

Other investigators focused specifically on antiphospholipid antibodies, which are reported to be found in aging people and diseases associated with aging.[257] In this study, anticardiolipin IgG levels were associated with AMD, supported by the findings of Morohoshi and colleagues which showed higher expression of anticardiolipin antibodies in nAMD compared with controls.[233, 257]

As described in Section 2.3.1, anti-CEP antibodies have also been investigated in association with AMD.[118, 117, 242]

2.4.4.3 Antibodies against pathogens

Infection by pathogens leads to increased antibody titers of the foreign pathogen.

Several infectious agents have been implicated in AMD, and we detail the antibodies against these pathogens in this section.

Chlamydia pneumoniae is an intracellular bacterial species that has been linked to atherosclerosis.[137] Since AMD involves inflammatory processes similar to atherosclerosis, the association of *Chlamydia pneumoniae* with AMD was explored. One small case-control study found support for this with increased antibody levels in AMD patients,[167] whereas 4 larger studies did not find evidence for a relation between anti-*Chlamydia pneumonia* antibodies and AMD.[183, 188, 227, 283]

The cytomegalovirus is another infectious agent that has been hypothesized to be associated with the pathogenesis of AMD, based on the relation between inflammatory processes induced by infection and the resulting vasculopathy.[227] Only 2 studies investigated this association. One found no evidence for an association,[90] whereas the other described higher levels of antibodies against cytomegalovirus in nAMD compared with controls and dry AMD.[227]

Another infectious agent possibly involved in the pathogenesis of AMD is *Helicobacter pylori*. Two studies have tested an association between antibodies against *Helicobacter pylori* and AMD but found no evidence for this, even when distinguishing between dry and neovascular AMD.[188, 227]

To summarize the most important findings regarding immune-related factors, involvement of the complement system in AMD is evident and complement activation products seem to be good biomarker candidates. Increased levels of inflammatory factors, such as CCL2 or CRP, have been frequently reported and support the notion that inflammatory processes underlie AMD. Yet, these are not specifically related to AMD and may therefore not be the best biomarker for clinical implementation. The use of multiplex assays for the simultaneous detection of multiple inflammatory markers (cytokines and chemokines) holds great promise, but additional data are required to determine their usefulness as AMD biomarkers. In addition, ARAs are also associated with AMD, but at present, it is unclear whether these autoantibodies play a direct role in the etiology of the disease or rather are the result of retinal damage. Further research is therefore necessary to determine if (specific) ARAs could be used as a biomarker for AMD.

2.5 LIPID METABOLISM/HOMEOSTASIS

Lipid metabolism is one of the major pathways involved in the pathogenesis of AMD as evidenced by genetic associations of lipid-linked genes *CETP*, *LIPC*, *ABCA1*, and *APOE*.[99, 101] Moreover, drusen, the major hallmark of AMD, consists of at least 40% lipids.[126, 350] In addition, as mentioned in Section 2.4.4, there are similarities in the pathogenesis of atherosclerosis and AMD.[368] Because lipids are important risk factors for atherosclerosis and CVD,[207] these might also be associated with AMD. Numerous studies have measured lipid levels in serum or plasma, and the results of these studies are summarized in Sections 2.5.1, 2.5.2, 2.5.3, 2.5.4. We focus on studies that reported associations with AMD and results from large population-based studies. A complete overview of all studies and references is provided in Supplementary Table 4.

2.5.1 Lipids

Cholesterol has multiple functions. It is required for building and maintaining cell membranes, is involved in cell signaling processes, and is a precursor molecule for synthesis of steroid hormones, bile acids, and vitamin D.[131]

The population-based Cardiovascular Health Study reported lower levels of total cholesterol in AMD patients, of which the majority had early AMD.[185, 217] Also in the Beaver Dam Eye Study, lower cholesterol levels were associated with development of early AMD in women,[182] and there was a trend for lower levels of cholesterol in nAMD[186]; a more recent analysis of the Beaver Dam Eye Study data, however, did not show an association between AMD and cholesterol levels.[183] In addition, 2 case-control studies described lower levels of cholesterol in AMD patients.[36, 267] In contrast, higher cholesterol was associated with AMD in 10 studies, although these were all case-control studies, and only half studied nAMD.[8, 57, 67, 88, 97, 109, 134, 152, 246, 342] The vast majority of studies (Supplementary Table 4), however, did not demonstrate a difference in cholesterol levels between AMD patients and controls, including a meta-analysis of 3 large population-based studies,[190] and several large population-based studies.[33, 37, 42, 50, 61, 73, 141, 143, 157, 160, 161, 176, 177, 184, 199, 261, 304, 306, 321, 331, 345, 354, 367,371]

Triglycerides are molecules that have a glycerol backbone connected to 3 fatty acids of variable length. Most studies did not report differences in triglyceride levels between AMD cases and controls (Supplementary Table 4). Lower triglyceride levels were reported in early AMD,[185, 371] nAMD,[219] and any AMD.[33, 177, 265, 285, 304] In contrast, 3

studies reported a higher level of triglycerides to be associated with AMD,[67, 235, 246] of these, 1 study included only women,[245] and 1 study found the association in women only.[235]

Phospholipids are another class of lipids and are an important component of cell membranes. In 3 studies, no association was found between phospholipids and AMD.[1, 40, 292]

2.5.2 Lipoproteins

Because of the insoluble nature of lipid molecules, lipoproteins are needed for transportation of lipids through the circulation. Five different lipoproteins exist, differing in their density and size: chylomicrons, very low–density lipoprotein, intermediate-density lipoprotein, LDL, and HDL.[255] Both HDL and LDL carry cholesterol between the liver and periphery.[131, 208, 265] The association between these 2 lipoproteins and AMD has been extensively studied.

For AMD, higher levels of LDL-C were found in several studies. Half of these studies found this association when comparing controls to nAMD,[109, 152, 154, 279, 342] others found an association in early AMD,[273] any AMD,[57, 67] and in women with dry AMD.[246] Almost all other studies, including multiple large population-based studies,[33, 37, 50,61, 184, 304, 331, 354, 371, 377] did not report an association between AMD and LDL-C (Supplementary Table 4). Only the Cardiovascular Health Study associated lower LDL-C levels with early AMD patients[185] and reported a trend toward lower levels in patients with any AMD.[217] Differences in results regarding LDL-C levels can be partly due to different measurement methods across studies, as it can either be measured directly, but more often is estimated using the Friedewald equation.[98]

Since HDL cholesterol (HDL-C) is inversely associated with CVD, one may have expected to also find this inverse association with AMD. Surprisingly, lower HDL-C levels were only described in a few studies in varying AMD stages; in late AMD,[279, 331] in women with dry AMD,[246] and in early AMD.[180] Increased HDL-C levels in AMD patients were present in multiple studies.[15, 36, 50, 61, 73, 141, 144, 161, 182, 184, 186, 265, 304, 345, 356, 376] It must be noted that most of these studies only found a weak association in a subgroup of AMD patients. Most of the studies did not describe significant differences in HDL-C levels (Supplementary Table 4).

Three studies evaluated non-HDL-C, which is calculated by subtracting HDL-C from total cholesterol. Two studies, including a large meta-analysis of 3 population-based studies, reported no association with AMD,[190, 265] whereas the third study found higher non-HDL-C to be associated with any AMD.[57]

Lipoprotein (a), Lp(a), is an LDL-like particle, which consists of apolipoprotein-B100 and apoliprotein-A. Its precise function is unclear, but higher levels of Lp(a) have been repeatedly associated with CVD.[82, 171] Contrarily, no association of Lp(a) levels with AMD or progression of AMD has been described so far.[1, 57, 83, 94,185, 246, 303]

2.5.3 Apolipoproteins

Apolipoproteins bind lipids to form lipoproteins that are responsible for lipid transport. They also function as enzyme cofactors and receptor ligands.[1] There are several classes of apolipoproteins. The overview presented in this section is restricted to apolipoprotein A1 (ApoA1), the major component of HDL-C, apolipoprotein B (ApoB), mostly found in LDL-C, and apolipoprotein E (ApoE), found in IDL-C and chylomicrons. Several investigations found an association between apolipoproteins and AMD or features of AMD.[1, 73, 94, 246, 265] The Pathologies Oculaires Liées à l'Age (POLA) study described ApoA1 to be associated with an increased risk of soft drusen[73] and also in the European Genetic Database (EUGENDA) cohort, higher levels of ApoA1 were associated with AMD, even after adjustment for genetic variants that influence lipid levels.[265] In contrast, one study reported a lower ApoA1 concentration in women with dry AMD.[246] This study also described a higher concentration of ApoB in dry AMD cases, which is in concordance with another study.[94] Higher ApoE levels were reported in advanced AMD compared with early AMD and control individuals; this difference could be due to a higher allelic burden of the *APOE* gene in these patients.[1] Other studies did not describe an association between ApoA1, ApoB, or ApoE and AMD.[57, 66, 83, 185]

2.5.4 Fatty acids

There are different types of fatty acids. PUFAs usually derive from phospholipids or triglycerides.[245, 254] The most commonly studied PUFAs in AMD are the omega-3 fatty acids DHA and eicosapentaenoic acid (EPA). Fish and other seafood are the main source of these omega-3 PUFAs.[219, 221] Animal and epidemiological studies have shown a lower risk for AMD in subjects with high dietary intake of omega-3 fatty acids.[21, 324] Also 2 interventional studies with omega-3 fatty acid supplementation have been performed;

the Age-related Eye Disease Study 2 showed no beneficial effect for omega-3 fatty acid supplementation,[3] whereas the Nutritional AMD Treatment 2 study showed a protective effect for DHA supplementation only in patient homozygous for the major allele (T) of the Y402H variant in the *CFH* gene.[222]

Considering omega-3 fatty acids as potential biomarkers, a number of studies investigated plasma or serum levels of these factors. In the Antioxydants, Lipides Essentiels, Nutrition et maladies Oculaires (ALIENOR), a population-based study, advanced AMD cases had lower plasma levels of α-linoleic acid and DHA compared with no or early AMD. In addition, lower plasma levels of EPA were associated with GA.[221] This is in line with baseline measurements performed in the Nutritional AMD Treatment 2 study that showed that nAMD cases had lower EPA and DHA levels in red blood cell membranes and lower serum EPA.[219] On the contrary, smaller case-controls studies reported no effect or opposite effects for DHA, EPA, and α-linoleic acid.[165, 252, 254, 292] For plasma or serum levels of docosapentaenoic acid, another omega-3 fatty acid, no significant associations were described.[165, 221, 292]

Omega-6 fatty acids, arachidonic acid and linoleic acid, and omega-9 fatty acid, oleic acid, have also been measured. A small case-control study found lower levels of linoleic acid and oleic acid, and higher levels of arachidonic acid in the membranes of erythrocytes of AMD patients.[254] In line with these findings, a recent study reported higher serum arachidonic acid in nAMD.[252] Two larger case-control studies, however, did not show different levels of these omega-6 and omega-9 fatty acids.[165, 292]

Regarding saturated fatty acids (which are single bonded), lower levels of palmitic acid in erythrocytes of AMD patients were reported in a small, case-control study,[254] although systemic levels were not different between cases and controls.[165, 254] Also for stearic acid, no association with AMD was detected.[165, 254]

Evidence for the involvement of lipids in AMD comes from epidemiologic, molecular, and genetic studies, but the exact role of systemic lipid levels is not yet clear. These studies are complicated by high variability of lipid and fatty acid levels in general and are potentially further confounded by the use of medication and/or dietary intake, including supplements. Although a combination of factors could constitute a risk profile that may be linked to the development and progression of AMD, it is unlikely that these factors individually could act as proper biomarkers for the disease.

2.6 EXTRACELLULAR MATRIX

Remodeling of the ECM plays a role in the pathogenesis of AMD.[158, 241] Drusen development, as well as alterations of Bruch membrane[52, 59] and infiltration of immune cells, relate to a balance between structural tightness or looseness of the extracellular environment. The constant remodeling of the ECM is carefully regulated by matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases.[236] Dysregulation of MMPs and/or tissue inhibitors of metalloproteinases could lead to ECM changes seen in AMD, and therefore, these are potentially useful biomarkers for AMD.

Genetic variations in several ECM-related genes are associated with AMD[99, 101, 275]; however, only few studies have measured plasma or serum levels of MMPs and tissue inhibitors of metalloproteinases.[45, 46, 120, 188, 381] An overview of the studies and references is provided in Supplementary Table 5. Upregulation of MMP9 in plasma was associated with AMD in 1 study[45]; however, 2 other studies could not replicate these findings.[120, 381] No association was found for serum MMP1 levels[120, 381] or MMP2 in serum or plasma.[45, 120, 381]

All 3 studies were limited because of small samples sizes and the measurement techniques used. Moreover, in these studies, both the proenzyme and active forms were measured together. Increased immunoactivity of MMPs does not necessarily mean an increase in enzymatic activity. Other measurement techniques are required to measure MMP activity more reliably, and larger future studies are needed to elucidate the potency of MMPs as biomarkers for AMD.

One of the main constituents of the ECM in Bruch membrane is elastin.[241] Elastin, in combination with other proteins of the ECM,[348] provides strong and long-lasting elasticity to the Bruch's membrane. The elastin layer degrades with age, however, and elastin metabolism may contribute to AMD where there is frequently thinning and fragmentation of the elastic layer,[52] especially in relationship to choroidal neovascularization.[31, 133] There is also evidence for abnormal systemic elastin metabolism in AMD. Patients with nAMD had significantly increased susceptibility to elastolysis in the skin.[31] Patients with nAMD had significantly higher levels of serum elastin-derived peptide levels,[318] probably due to the aforementioned elevated levels of MMPs in serum.[45] Apart from elevated elastin peptide fragment levels, sera from patients with AMD contain specific autoantibodies against elastin and it has been

suggested that the IgG/IgM ratio for elastin, and other, autoantibodies might allow monitoring the progression of AMD.[233] Therefore, analyzing elastin degradation products or autoantibody levels or ratios might be useful tools as biomarkers, at least for nAMD.

2.7 DIETARY FACTORS

Known risk factors for AMD include dietary factors, such as low intake of antioxidants. Some vitamins are antioxidants, whereas others act as cofactors for enzymes involved in ROS clearance,[333] as detailed in Section 2.7.1. Trace elements have also been hypothesized to be involved in the pathogenesis of AMD and are described in Section 2.7.2. Another marker influenced by diet is serum albumin; this is considered to be an indicator of nutritional status and inflammation and is discussed in Section 2.7.3. In addition, diet is also an important source for fatty acids and carotenoids both related to AMD. These are described in Sections 2.3.4.2, 2.5.4, respectively. A complete overview of the studies and references is provided in Supplementary Table 6.

2.7.1 Vitamins

Vitamin C can act as an ROS scavenger, and it mediates reactivation of vitamin E.[333] When vitamin C hydrolyzes and reactivates vitamin E, the molecule itself is inactivated, and hydrolysis by GSH can reactivate vitamin C (Figure 1).[258] Lowered levels of vitamin C result in less vitamin E conversion to its active form. In addition, vitamin C itself cannot fulfill its antioxidant function, and as a consequence ROS production will rise.[258] Vitamin C levels were found to be lower in AMD patients than those in controls[311] and lower in advanced versus early AMD[313]; however, most studies do not report an association between vitamin C and AMD.[31, 72, 87, 88, 360, 375]

Vitamin E is anchored in the plasma membrane and prevents lipid peroxidation.[333] Lower levels of serum vitamin E in AMD patients were reported.[25, 214, 313, 360] However, associations with vitamin E were not conclusive because no difference in vitamin E levels has been found in several studies.[31, 40, 72, 87, 88, 224, 292, 311, 322, 339]

One study reported lower levels of vitamin A in patients with nAMD.[384] However, most studies did not find a significant association between vitamin A levels and AMD.[31, 72, 88, 224, 292, 313, 360]

B vitamins are essential molecules in homocysteine metabolism and synthesis of methionine. Both vitamin B9 (folate) and B12 (cobalamin) act as cofactors to convert homocysteine into methionine.[294] In AMD patients, lower serum levels of vitamin B12 were detected compared with controls.[113, 168, 284] These results were not consistently replicated, as equal levels of serum vitamin B12 in patients and controls have also been described.[132, 247] Folate levels were similar between controls and AMD patients in all studies.[113, 132, 168, 183, 247,284]

Vitamin D can be produced in the dermis upon sunlight exposure or can be obtained through diet. For its activity, the molecule has to be converted into its active form in the liver and kidney before it can regulate uptake of nutrients such as iron, calcium, magnesium, and zinc.[244] There are inconsistent results for vitamin D levels in AMD patients. They have been described to be higher,[177] lower,[150, 259] or not associated with the disease.[50, 62,111, 226, 234, 261, 316]

2.7.2 Trace elements

Trace elements are required by the human body in very low concentrations for proper physiological functioning; however, deficiency or excess amounts may be harmful.[27]

Iron is essential for retinal functioning, as phototransduction is dependent on iron-containing enzymes. Accumulation of iron, however, can be harmful. Iron can convert hydrogen peroxide (H_2O_2) into highly reactive ROS and thereby enhance oxidative stress.[323] Cadmium can also increase ROS formation[361] and mercury can decrease oxidant defense mechanisms,[140] both leading to increased oxidative stress. In contrast, manganese, copper, and zinc contribute to antioxidant activity as they are cofactors for the antioxidant enzyme SOD.[333, 362] GSHP is dependent on the presence of the essential heavy metal selenium.[17] In addition, copper and zinc are able to stabilize proteins, reducing their vulnerability to oxidation[362] but can also lead to pathological aggregation or even precipitation of proteins.[237, 238, 239] Both zinc and manganese can reduce uptake or accumulation of toxic cadmium.[293]

Several studies reported elevated cadmium levels in blood,[50, 176, 262, 366] aqueous humor,[163] and urine of AMD patients.[366] Measurement of cadmium levels in blood might represent only recent cadmium exposure, whereas urinary cadmium reflects long-term exposure to cadmium and might therefore be a more accurate biomarker. A study comparing both blood and urinary cadmium levels did not show an association with AMD in the total study group; however, when stratified for smoking status,

increased urinary cadmium levels were associated with AMD in smoking individuals, suggesting a smoke-related association of cadmium with AMD.[81] Lead levels were elevated in serum and urine of both early and advanced AMD,[50, 262, 366] and 1 study reported an association between lead and AMD only for women.[143] Levels of mercury were only elevated in patients with advanced AMD.[50, 262]

Selenium was in general not associated with AMD.[87, 88, 163] One study found a borderline significant association with AMD,[339] and another measured significantly lower levels of selenium in nAMD patients.[216] Conflicting results are reported for levels of iron,[31, 163, 369] copper,[40, 163] manganese,[163, 262] and zinc.[25, 88, 163, 262, 313]

2.7.3 Albumin

Albumin is essential for maintenance of plasma colloid oncotic pressure, acts as a plasma binding protein, and also has antioxidant activity.[202] In addition, albumin is one of the most common proteins found in drusen.[63] A few studies measured serum albumin in AMD patients and controls. Two case-control studies did not show a significant association between serum albumin and AMD.[31, 88] The population-based Cardiovascular Health Study and Beaver Dam Eye Study did report significantly lower serum albumin levels in early and neovascular AMD, respectively.[185, 187] A more recent nested case-control study within the Beaver Dam population further analyzing these data could not confirm decreased albumin levels in AMD.[183]

Taken together, because of the highly variable diet between subjects, and varying levels of dietary factors within subjects based on fasting state, assessment of the role of these dietary factors as biomarkers in AMD remains difficult. Dietary intake and/or supplementation of antioxidants and vitamins, however, have therapeutic benefit. The Age-related Eye Disease Study trial, one of the largest investigations into vitamin supplementation in AMD, focused on daily supplementation with vitamin E, vitamin C, β-carotene, and zinc and demonstrated a lower chance of advanced AMD development in subjects taking these supplements.[4] In the Age-related Eye Disease Study 2, an improved formula was evaluated and β-carotene was replaced by lutein/zeaxanthin because of the increased risk of lung cancer in smokers.[3, 5]

Regarding trace elements, toxic heavy metals (such as lead, mercury, and cadmium) are mainly associated with an increased risk of AMD, whereas essential heavy metals (e.g., zinc and manganese) seem to protect against the development of AMD. For most trace elements, there are only a limited number of studies available in the public domain to

date, and further research is required to assess their potential role as a biomarker or as protective supplement.

2.8 HORMONES

In this section, we discuss the few hormones that have been investigated in relation to AMD: leptin, melatonin, and dehydroepiandrosterone sulfate (DHEAS). A complete overview of the studies and references is provided in Supplementary Table 7.

2.8.1 Leptin

Because AMD is a multifactorial disease in which dietary factors and body mass index also play a role in the disease mechanism, it has been suggested that the principal hormone involved in food intake behavior, leptin, may be associated with AMD. Two studies support this theory; both showed a reduction in serum leptin levels in AMD patients compared with controls.[85, 306] After controlling for potential confounders, including smoking, body mass index, blood pressure, and HDL-C, the association remained significant, which suggests that mechanisms other than body fat underlie the relationship between leptin levels and AMD.[306] The third study did not observe a difference in leptin levels in patients versus control individuals.[124]

2.8.2 Melatonin

Melatonin has strong antioxidative capacities, is expressed in the retina, and expression levels decrease during aging.[173, 276, 277] Two studies investigated the levels of melatonin in AMD. One showed elevated blood levels of daytime melatonin in pseudophakic AMD patients.[296] The second study analyzed the major metabolite of melatonin in urine, 6-sulfatoxymelatonin, and described lower levels in AMD.[286] Comparing the 2 studies is difficult because of the differences in methodology and fluid matrix analyzed, so additional experiments linking melatonin and AMD are necessary.

2.8.3 Dehydroepiandrosterone sulfate

DHEAS is a sulfate ester of DHEA, which is an endogenous steroid hormone synthesized from cholesterol in the adrenal glands and serves as precursor molecule for sex steroids, androgen and estrogen.[212] It has been suggested that DHEAS has antioxidant effects.[212, 330, 342] In addition, the DHEAS level in blood decreases with age.[23, 212, 330] Since

both oxidative stress and aging are important risk factors for AMD,[59] the question arises whether DHEAS and AMD could be correlated. Three studies investigated the association between AMD and DHEAS, all with different outcomes; higher levels of DHEAS were reported in women with early AMD,[69] another study described low DHEAS in both dry and neovascular AMD cases,[330] and a third study did not find an association between nAMD and controls.[342]

In summary, only a limited amount of studies assessing hormones in AMD have been performed with inconclusive results and do not seem to be reliable biomarkers for AMD at this point in time.

2.9 FACTORS RELATED TO COMORBIDITIES

AMD has been suggested to share risk factors or coexist with other diseases, such as kidney disease, diabetes mellitus, and Alzheimer's disease. Factors related to these comorbidities are discussed in Sections 2.9.1, 2.9.2, 2.9.3, respectively. Although AMD has not been associated with liver disease before, some studies investigated factors related to liver function and these are described in Section 2.9.4. A complete overview of the studies and references is provided in Supplementary Table 8.

2.9.1 Kidney disease

Several studies have suggested overlapping risk factors between AMD and kidney diseases.[77, 189, 203, 356] A number of large, often population-based, studies have not only investigated kidney function, such as glomerular filtration rate, but also markers that can be measured in serum/plasma like creatinine and cystatin-C. In the Beaver Dam Eye Study, serum cystatin-C was associated to the incidence of early AMD and nAMD.[189] In the Multi-Ethnic Study of Atherosclerosis, this association was only found when the highest deciles of cystatin-C were compared with other deciles with prevalence of early AMD.[51] In the Hatoyama study, no association between cystatin-C and AMD was found.[15]

Several large studies investigated creatinine in patients, but no clear association between serum creatinine and AMD was found. Two reports from the Korean National Health and Nutrition Examination Survey describe a significant difference between AMD patients and controls, but after adjustment for other variables, no

significant association was found.[50, 261] The remainder of the studies, including large population-based studies such as the Multi-Ethnic Study of Atherosclerosis and the Singapore Malay Eye Study, did not find any association between serum creatinine and AMD.[31, 33, 37, 150, 152, 153, 247]

Another indicator of renal health is blood urea nitrogen, but also for this factor, no link was established with AMD.[31, 50, 189, 261]

2.9.2 Diabetes mellitus

Although some cardiovascular risk factors, such as smoking, have been consistently related to AMD, there are conflicting results for an association between diabetes mellitus and AMD.[43] Several studies, mostly population-based, measured glycated hemoglobin and glucose as indicators for the presence of diabetes mellitus. Only one study found lower levels of glucose in advanced AMD,[199] but none of the other studies described an association of either markers with AMD.[31, 33, 37, 73, 88, 143, 152, 153, 160, 176, 321, 371, 377] Several studies, all reports from the Korean National Health and Nutrition Examination Survey, reported lower glycated hemoglobin levels in AMD[50,143, 176, 177, 199]; however, studies from other cohorts detected no difference.[33, 37, 160, 342, 380]

2.9.3 Alzheimer's disease

Similar to AMD, the prevalence of Alzheimer's disease increases with age. This neurological disorder is characterized by amyloid plaques in the brain, with the main component being amyloid beta (Aβ).[16] In AMD, 2 studies identified Aβ as a component of drusen.[12, 75] In addition, Aβ might trigger activation of the complement cascade in AMD.[159] Several isoforms of Aβ with different amino acid lengths exist; in this section, we discuss the most common isoforms: Aβ1-40 and Aβ1-42.

A small, case-control study did not show different levels of Aβ1-42 between controls and either dry or neovascular AMD[247]; however, 2 more recent case-control studies showed significantly higher Aβ1-42 peptide levels in AMD patients.[120, 124] Also after correction for age, Aβ1-42 was significantly associated with AMD, and there was a trend toward increasing levels of Aβ with increasing disease severity.[120] An association of AMD with Aβ1-40 in these studies was less clear. A significant upregulation was described in one study in nAMD only,[120] whereas the other study did not report a difference between nAMD patients and controls.[124]

2.9.4 Liver function

So far, to our knowledge, no study has focused specifically on liver function and AMD. In a few studies, indicators of liver function have been reported as part of a routine blood examination with no associations between lactate dehydrogenase, aspartate transaminase, or alanine transaminase and AMD.[31, 50, 285]

For hepatitis B surface antigen on the other hand, an association was described in several Korean studies, a country where hepatitis B is still endemic.[50, 261, 285] In these studies, hepatitis B surface antigen carrier status was positively associated with AMD. Hepatitis B surface antigen has been detected in subretinal fluid, and it is hypothesized these individuals are therefore at increased risk for uveoretinal pathology, such as AMD.[261, 285]

In conclusion, despite coexistence and overlapping risk factors with AMD, biomarkers for kidney disease, diabetes mellitus, and liver disease discussed here do not seem good biomarker candidates for AMD. As an exception, Aβ could potentially be a marker of disease progression; however, larger prospective studies are required to confirm these findings. In addition, also in terms of a potential new drug target, further evaluation of this biomarker in AMD seems worthwhile, as promising anti-Aβ therapies are being developed for Alzheimer's disease.[16]

2.10 HYPOTHESIS-FREE TECHNIQUES

In the past decade, many advanced high-throughput omic technologies have been developed. These technologies enable us to analyze large numbers of markers at the same time in an untargeted and unbiased manner. Here, we discuss several omic technologies in association with AMD (Figure 3): proteomics (Section 2.10.1), metabolomics (Section 2.10.2), and epigenomics (Section 2.10.3). Expression of circulating microRNAs can also be measured using high-throughput techniques; these are described in Section 2.10.4.

2.10.1 Proteomics

The field of proteomic research uses mass spectrometry, or variations to this technique, to determine the nature of peptides or proteins in various tissues or other biological

samples. The advantage of proteomic research is that it delivers results that are unbiased by preconceived notions or hypotheses. Within the field of AMD, proteomics has been used in a number of investigations, and several have been successful in showing particular proteomic signatures in plasma, vitreous, and aqueous humor from AMD patients when compared with controls.

A small study by Kim and colleagues identified 154 proteins in aqueous humor of 9 nAMD patients and 8 cataract controls.[178] In this study, 7 potential biomarker candidates were selected for further analysis: ceruloplasmin, PEDF, plasma protease C1 inhibitor, TGF-β1, clusterin, cathepsin D, and cystatin D. The relative abundances of TGF-β1, plasma protease C1 inhibitor, ceruloplasmin, and PEDF were shown to be significantly higher in AMD samples compared with controls. Another small study, collecting and profiling aqueous humor of 6 nAMD patients and 6 cataract controls, found 68 proteins to be differentially expressed.[372] Only 9 proteins were identified in both studies, among which were some that were related to AMD previously (CCL24 and complement FI), lipocalin-1 and several members of the crystallin family. These crystallins, known for their chaperone function, may also be involved in protein-protein interaction, prevention of apoptosis, and inhibition of inflammation among others.[170] Lipocalin-1 concentrations were quantified using enzyme-linked immunosorbent assay, and levels were significantly elevated in the aqueous humor of nAMD patients.

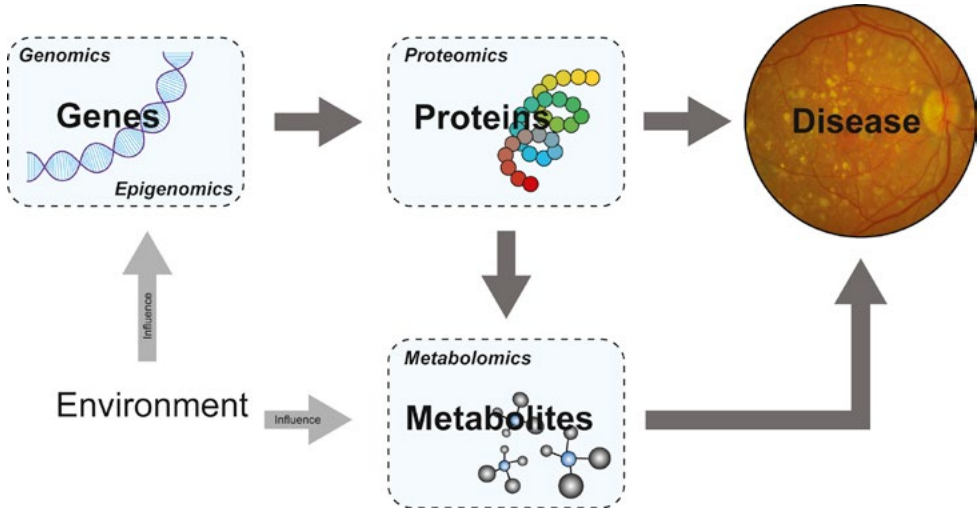

Figure 3. Omics in age-related macular degeneration.

A third small study performed a focused proteomic analysis on protein members of the ubiquitin pathway.[201] Difference in expression of 6 proteins in aqueous humor of 2 AMD patients compared with 2 controls was reported. This included the 26S proteasome non-ATPase regulatory subunit 1 (Rpn2), a protein that is also present in plasma. Rpn2 was therefore selected as potential AMD biomarker and liquid chromatography-multiple reaction monitoring mass spectrometry of another 15 aqueous humor samples showed a relative increase of Rpn2 in nAMD patients.

Kang and colleagues analyzed aqueous humor samples of 26 treatment naive patients with nAMD and 18 controls.[169] By comparing expression profiles in exosomes of aqueous humor and cultured RPE cells, 6 candidate proteins were selected for verification in an independent sample set by liquid chromatography-multiple reaction monitoring mass spectrometry: actin, myosin-9, heat shock protein 70, cathepsin D, cytokeratin 8, and cytokeratin 14. Of these, cytokeratin 8 showed the highest area under the curve value (0.929), suggesting that it is a strong predictor for AMD. Although cytokeratins were not previously reported in other proteomic analyses in AMD and might be valuable markers to further investigate, it is disputable whether they could qualify as manageable biomarkers. Cytokeratins are abundant contaminants in laboratories,[209] so careful replication of these findings in other laboratories is warranted.

One other study investigated in a targeted manner the involvement of Wnt modulators in aqueous humor and found that WNT inhibitory factor 1 (WIF-1) and Dickkopf-related protein 3 (DKK-3) were upregulated in nAMD.[260]

In a study of 73 nAMD patients and 15 controls, a large set of proteins were detected in vitreous humor, of which 19 were upregulated in nAMD patients.[194] Bioinformatic analyses suggested enrichment of the complement and coagulation cascades, as well as markers involved in arachidonic acid metabolism. Of the 19 proteins, 5 were randomly selected for Western blot validation; alpha-1-antitrypsin reached statistical significance, whereas ApoA1 and transthyretin showed a nonsignificant increase in AMD. These findings need validation in a larger sample set.

Nobl and colleagues investigated vitreous samples of 108 nAMD patients and 24 controls, distributed over a discovery and validation set, and discovered 101 different proteins.[243] Using a closed testing procedure, they focused on 4 differentially expressed proteins as candidate AMD biomarkers: clusterin, opticin, PEDF, and

PH2D, which were increased in nAMD compared with controls, except for opticin, which was reduced. Upregulation of PEDF and PH2D in nAMD was described previously.[178, 194] Clusterin and PEDF remained significantly increased in nAMD after validation and correction for multiple testing in an independent sample set using enzyme-linked immunosorbent assay.

There have been limited plasma proteomic studies. Xu and colleagues found 28 clinically relevant proteins to be altered in AMD patients (N = 24) compared with healthy volunteers (N = 6),[370] but further investigation of these plasma proteins is necessary to validate these findings. In addition, 2 studies using proteomic profiling of the same data set identified 3 potential AMD biomarkers: vinculin, phospolipid transfer protein, and mannan-binding lectin protease-1.[175, 179] In general, proteomics of plasma or serum is a great analytical challenge due to the dominant fraction of highly abundant proteins, which have effectively prevented the discovery of novel proteomic biomarkers in these fluids in the past. Therefore, improved technologies are needed. Fortunately, some progress has been made using quantitative shot-gun proteomics, recently.[108]

2.10.2 Metabolomics

Metabolomic studies use mass spectrometric technologies or nuclear magnetic resonance spectroscopy to measure derivatives of metabolism. The technique offers a snapshot of the physiological state of an organism at the level of body fluids (urine, tears, serum, and plasma), cells or even tissues. Metabolomic analysis of AMD has great potential to uncover novel pathways in the disease that are reflective of the interaction between the genetic blueprint of individual and environmental factors that influence the metabolites (e.g., diet and smoking). To date, only one metabolome-wide study was conducted in plasma samples of 26 nAMD patients and 19 controls. Pathway analysis pointed toward involvement of tyrosine metabolism, urea metabolism, and vitamin-D–related metabolism.[253]

2.10.3 Epigenomics

Although it is clear that both genetic components as well as environmental elements contribute to the risk of developing AMD, it is less clear how these 2 systems interact. This interaction is the domain of epigenetics, induced changes in the expression levels of genes controlled by outside influences. Epigenetics is a broad term, encompassing many possible regulatory mechanisms of gene expression. One type of epigenetic mark

that has been explored in a number of studies is the difference in DNA methylation patterns between cases and controls.

Epigenetic changes can be observed in peripheral blood leukocytes, which are relatively easy to obtain. One study showed a decrease in methylation near the IL17RC promotor region, suggesting that this could serve as a potential biomarker for AMD.[355] However, the finding could not be validated by an independent study with a sufficiently powered study design.[249]

Based on these results, and also because epigenetic mechanisms are likely to be tissue specific, the relationship between DNA methylation patterns in peripheral blood and retinal tissue was investigated in a recent study.[250]Although no epigenome-wide association peak was observed, the study did report consistent methylation changes across multiple samples near the *ARMS2* locus and near the protease serine 50 (*PRSS50*) gene.

Despite a limited sample size, the results provided some evidence that methylation patterns in blood leukocytes could serve as proxies for retinal changes, implying that such studies could deliver additional biomarkers for AMD.[250]

2.10.4 Circulating microRNAs

A microRNA (miRNA) is a small noncoding RNA molecule that regulates gene expression after transcription, thereby influencing biological processes. These miRNAs are present in circulation and could potentially serve as biomarkers.[229] Because we focus on compounds found in body fluids, only the studies that investigate circulating miRNAs (cmiRNAs) in serum or plasma are described here.

In a small study by Ertekin and colleagues,[84] plasma samples of 33 nAMD patients and 31 controls were analyzed for the expression of 384 miRNAs. They found 16 miRNAs to be differentially expressed between the 2 groups and additionally discovered 10 miRNAs to be only expressed in nAMD patients.

Grassmann and colleagues identified 203 cmiRNAs in serum, of which 3 (hsa-mir-301-3p, hsa-mir-361-5p, and hsa-mir-424-5p) were significantly altered in nAMD patients (N = 129) compared with control individuals (N = 147).[114] No significant association was found in GA patients (N = 59), suggesting different mechanisms for advanced AMD subtypes. Pathway analysis of the genes that are likely regulated by the altered

cmiRNAs implicated the mTOR and TGF-β pathways in nAMD and knockdown of these cmiRNAs in vitro resulted in increased angiogenesis but only significantly for hsa-mir-361-5p.

Szemraj and colleagues also reported significant differences in cmiRNA profiles between dry and neovascular AMD patients.[328] In this study, serum expression levels of 377 miRNA genes in 300 AMD patients (150 nAMD/150 dry AMD patients) and 200 control individuals were analyzed. This study identified 31 differentially expressed miRNAs between patients and controls, including 2 of the 3 previously associated[114] cmiRNAs (hsa-mir-301-5p and hsa-mir-424-5p). Of the differentially expressed miRNAs in this study, 5 were significantly different between patients with dry and neovascular AMD. In addition, the correlation between these miRNAs and expression of VEGF and VEGFR2 was assessed, and it was suggested that miRNA Let-7 is implicated in the neoangiogenesis in nAMD.

So far, limited studies on miRNA profiling in AMD have been performed and results need to be replicated in larger studies; however, these initial findings emphasize the potential of cmiRNAs as biomarkers in AMD.

In general, studies using hypothesis-free techniques demonstrate proof of concept that omic analyses are able to identify novel biomarkers for AMD; however, more are needed to validate results and to confirm the clinical utility of these biomarkers.

2.11 CONCLUSION AND FUTURE DIRECTIONS

In summary, numerous compounds have been analyzed in relation to AMD. However, only a few of these have potential as AMD biomarkers. The most promising biomarker candidates belong to the oxidative stress pathway, the complement system, and to a lesser extent, lipid metabolism. Finally, the use of hypothesis-free techniques in biomarker detection holds great promise. For summarized findings regarding factors belonging to the other biological pathways described in this review, we refer to the closing paragraphs of the respective chapters. As of yet, none of the biomarkers that we have reviewed here are used clinically.

Many studies reported decreased antioxidant levels and elevated levels of oxidized proteins or lipids indicating oxidative stress in AMD. MDA is often used as a marker for lipid peroxidation, and increased levels of MDA have been very consistently observed in both wet and dry AMD (11 of 11 studies, Section 2.3.1). In addition, most studies reported higher levels of homocysteine, an intermediate in the oxidative stress pathway, in AMD (12 of 18 studies, Section 2.3.3). Besides dysregulation of the oxidative stress pathway, many studies indicate the involvement of the complement system in AMD. Products of complement activation and levels of complement activation—described by the ratio of C3 and its degradation product C3d (C3d/C3)—were repeatedly associated with AMD (Section 2.4.1). In addition, there is clear involvement of lipids in AMD from genetic and molecular studies; however, the role of systemic lipids in AMD is not fully elucidated, and therefore, they are not yet applicable as robust biomarkers for the disease.

In general, many inconsistencies exist between studies evaluating biomarkers and their association with AMD. The contradicting results are difficult to interpret due to a variety of differences between studies, including methodological differences (fasting vs nonfasting blood), different populations (Caucasian/Asian/Mediterranean) with different dietary habits, different study designs, different analytical methods, and correction factors, but also types of AMD included in the studies. It must be noted that compiling and comparison of data deriving from different sources represent a major limitation. Therefore, large well-conducted prospective studies are needed to further clarify these results.

Although AMD represents a phenotype restricted to the eye, many studies have investigated systemic markers in relation to AMD; however, because of the presence of the blood-retinal barrier, biomarkers might be only locally dysregulated inside the eye without a measurable systemic effect. In addition, some compounds are differently expressed between tissues, leading to different results when analyzing different matrices. One might therefore argue to measure markers only locally; however, because of the invasive character and accompanying ethical issues, systemic markers are preferred for implementation as clinical biomarkers.

Until now, most studies have targeted specific single biomarkers in a candidate-driven approach. Omic studies with an unbiased view are heavily outnumbered. Future biomarker research should therefore combine hypothesis-free as well as candidate-driven approaches. Quantitative analytical approaches applied in an untargeted and

targeted fashion, such as metabolomic or proteomic studies, are necessary to identify novel biomarker candidates. Once validated as robust and reliable markers, they can offer more insights into the etiology and pathogenesis of AMD and support prediction, diagnosis, stratification, monitoring of treatment, and drug development for AMD.

Other biomarker types in AMD such as genetic factors, imaging biomarkers, or visual function measurements are currently of key importance for proper clinical diagnosis, stratification, and treatment of AMD. In the future, these established clinical examinations and diagnostic tests may well be applied in combination with molecular biomarkers, an area which is still in a nascent stage.

2.12 METHODS OF LITERATURE SEARCH

A review of literature was performed through a thorough PubMed search in November 2015. We used the following keywords and their synonyms in various combinations: age-related macular degeneration, serum, plasma, blood, urine, tear, aqueous, and vitreous. No limitations were set for the time range covered by our search, and therefore, all articles published until our search were included.

All abstracts were screened for relevance and full texts of the selected articles were studied. We included only articles written in English. Articles cited in the reference lists of articles obtained through this search were also included whenever relevant. Animal, ex vivo, and in vitro studies were excluded. To include the most recent developments before submission, the search was repeated in June 2016. An overview of our selection process is detailed in Figure 4.

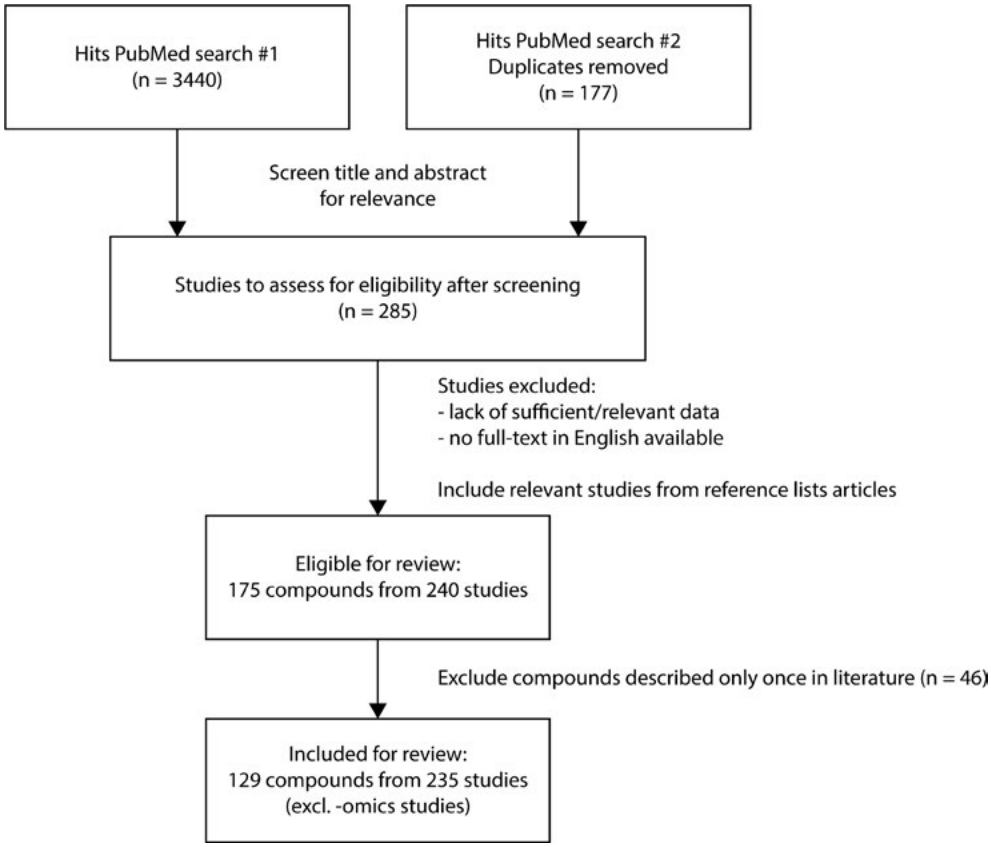

Figure 4. Flow diagram of literature search. The screening and selection process of studies included for this review is depicted in the flow diagram. After the final article selection, all described compounds in these studies were grouped based on their common biological function or pathway, and results were discussed accordingly. Of note, compounds that were only described once in literature were not mentioned in this review to reduce the effect of selective reporting.

2.13 SUPPLEMENTARY DATA

Supplementary table 1. Factors involved in neovascularization

	Reference	Up/down/ no difference	Type of AMD	Cases (n)	Controls (n)	Study design	Matrix	Comment
Vascular Endothelial Growth Factor (VEGF)	(Lip et al., 2001)	Up	Any AMD	78	25	Cross-sectional	Plasma	
	(Holekamp et al., 2002)	No difference	Advanced: Neovascular	9	12	Case-control	Vitreous	
	(Tong et al., 2006)	Up	Advanced: Neovascular	12	10	Prospective Case-control	Aqueous	
	(Tsai et al., 2006)	Up	Early AMD/ Advanced: neovascular	17/ 60	42	Case-control	Plasma	VEGF levels were higher in nAMD vs. early AMD
	(Mo et al., 2010)	No difference/ No difference/ No difference	Early AMD/ Advanced: GA/ Advanced: Neovascular	39/ 20/ 19	18	Case-control	Serum	
	(Machalinska et al., 2011b)	No difference	Advanced: Neovascular	29	38	Case-control	Plasma	
	(Haas et al., 2011b)	No difference	Advanced: Neovascular	66	66	Case-control	Serum	
	(Huber and Wachtlin, 2012)	No difference	Advanced: Neovascular	12	11	Case-control	Vitreous	
	(Carneiro et al., 2012)	No difference	Advanced: Neovascular	43	19	Case-control	Plasma	
	(dell'Omo et al., 2012)	Up	Advanced: Neovascular	29	14	Prospective Case-control	Aqueous	VEGF levels were higher in type 3 neovascularizations compared to controls and type 1/2
	(Grierson et al., 2013)	Up	Advanced: Neovascular	31	10	Case-control	Plasma	
	(Wang et al., 2014b)	No difference	Advanced: Neovascular	32	12	Case-control	Plasma, serum	
	(Zehetner et al., 2014)	No difference	Advanced: Neovascular	30	12	Prospective Case-control	Plasma	Significant positive correlation with PDGF-B
	(Gu et al., 2014)	No difference	Advanced: Neovascular	39	39	Case-control	Serum	
	(Scotti et al., 2014)	No difference	Advanced: Neovascular	23	20	Case-control	Plasma	
	(Ambreen et al., 2015)	Up	Any AMD	90	100	Cross-sectional Case-control	Serum	VEGF was sign higher in wet vs dry AMD
	(Enders et al., 2015)	No difference	Advanced: Neovascular	61	68	Prospective observational	Plasma	Trend towards downregulation (P=0.06)
	(Goncalves et al., 2015)	No difference	Advanced: Neovascular	50	30	Case-Control	Serum	Patients received treatment, results might reflect treatment efficacy

	(Sakurada et al., 2015)	No difference	Advanced: Neovascular	18	20	Case-control	Aqueous	
Soluble VEGF receptor 1	(Huber and Wachtlin, 2012)	Up	Advanced: Neovascular	12	11	Case-control	Vitreous	
	(Owen et al., 2014)	No difference	Advanced: Neovascular	341	198	Family-based cohort	Serum	Only analyzed in discovery cohort (n=322)
	(Uehara et al., 2015)	No difference/ Down	Early AMD/ Advanced: Neovascular	53/ 97	56	Case-control	Serum	sVEGF receptor 1 was lower in nAMD than in early
PEDF	(Holekamp et al., 2002)	Down	Advanced: Neovascular	9	12	Case-control	Vitreous	
	(Tong et al., 2006)	Up	Advanced: Neovascular	12	10	Case-control	Aqueous	
	(Huber and Wachtlin, 2012)	Down	Advanced: Neovascular	12	11	Case-control	Vitreous	
TGF-beta	(Guymer et al., 2011)	Up/ No difference/ No difference	Early AMD/ Advanced: GA/ Advanced: Neovascular	51/ 19/ 33	54	Cross-sectional	Urine	No correlation between serum and urinary TGF-beta levels
	(Bai et al., 2014)	Up	Advanced: Neovascular	14	12	Case-control	Vitreous	
Fibrinogen	(Inhoffen and Nussgens, 1990)	No difference	Advanced: Neovascular	35	35	Case-control	Blood	
	(Eye-Disease-Case-Control-Study-Group, 1992)	Up	Advanced: Neovascular	421	615	Case-control	Serum	
	(Smith et al., 1998)	No difference/ Up	Early AMD/ Advanced: Any	240/ 72	3342	Population-Based Cross-sectional	Plasma	Blue Mountains Eye Study
	(Lip et al., 2001)	Up	Any AMD	78	25	Case-control	Plasma	
	(Klein et al., 2003a)	No difference	Early AMD	366	1995	Population-Based Cohort	Serum	Cardiovascular Health Study
	(Dasch et al., 2005)	Up/ Up	Early AMD/ Advanced: Any	422/ 270	181	Case-control	Plasma	Muenster Aging and Retina Study
	(Schaumberg et al., 2007)	No difference	Any AMD	150	27537	Population-Based Longitudinal	Plasma	Women's Health Study, only women included in study
	(Tan et al., 2007)	No difference/ No difference	Early AMD/ Advanced: Any	?	?	Population-Based Cohort	Serum	Blue Mountains Eye Study, numbers are unclear from text (total n=2395)
	(Wu et al., 2007)	No difference/ No difference	Early AMD/ Advanced: Any	159/ 38	433	Population-Based Cross-sectional Case-control	Plasma	Blue Mountains Eye Study
	(Klein et al., 2008)	No difference/	Early AMD/	235/	5623	Prospective Cohort	Plasma	Multi-Ethnic Study of Atherosclerosis

		No difference	Advanced: Any	29				
	(Michalska-Malecka et al., 2008)	Up	Any AMD	52	42	Case-control	Plasma	
	(Wang et al., 2008)	No difference	Any AMD	278	557	Population-Based Case-control	Serum	Blue Mountains Eye Study
	(Rudnicka et al., 2010)	No difference	Advanced: Any	81	77	Case-control	Blood	
	(Colak et al., 2012)	No difference	Any AMD	84	84	Case-control	Plasma	Subgroup analyses showed higher risk of AMD when fibrinogen > 3.8 g/l (P=0.019)
Plasminogen activator inhibitor I (PAI-1)	(Wu et al., 2007)	Up/ Up	Early AMD/ Advanced: Any	159/ 38	433	Population-Based Cross-sectional Case-control	Plasma	Blue Mountains Eye Study
	(Wang et al., 2008)	No difference	Any AMD	188	393	Population-Based Case-control	Serum	Blue Mountains Eye Study, conflict between text and table
	(Rudnicka et al., 2010)	No difference	Advanced: Any	81	77	Case-control	Blood	
	(Bertelmann et al., 2013)	No difference	Dry AMD/ Advanced: Neovascular	13/ 6	30	Case-control	Aqueous	PAI-1 was not detected in neither groups
Platelet count	(Inhoffen and Nussgens, 1990)	No difference	Advanced: Neovascular	35	35	Case-control	Blood	
	(Lip et al., 2001)	No difference	Any AMD	78	25	Cross-sectional	Blood	Borderline significant upregulation in AMD (P=0.055)
	(Klein et al., 2003c)	No difference/ No difference/ No difference	Early AMD/ Advanced: GA/ Advanced: Neovascular	?	?	Population-Based Cohort	Blood	Beaver Dam Eye Study, total n=3672, case-control ratios not presented
	(Klein et al., 2007a)	No difference/ No difference/ No difference	Early AMD/ Advanced: Neovascular/ Advanced: GA	866/ 39/ 14	3369	Observational	Blood	Women's Health Initiative Sight Examination, only women included
	(Roh et al., 2008)	Down	Any AMD	235	9082	Case-control	Serum	Yonsei Eye Study, not significant after adjustment for age
	(Klein et al., 2010)	No difference	Early AMD	96	2714	Cross-sectional	Blood	
	(Cho et al., 2014)	Down/ No difference	Any AMD/ Advanced: Any	584/ 55	7315	Population-Based Cross-sectional	Serum	KNHANES, only significant in univariate analyses, not in multivariate
Von Willebrand factor (VWF)	(Lip et al., 2001)	Up	Any AMD	78	25	Case-control	Plasma	
	(Wu et al., 2007)	No difference/ No difference	Early AMD/ Advanced: Any	159/ 38	433	Population-Based Cross-sectional Case-control	Blood	Blue Mountains Eye Study

(Wang et al., 2008)	No difference	Any AMD	188	393	Population-Based Case-control	Serum	Blue Mountains Eye Study, Conflict between text and table
(Rudnicka et al., 2010)	No difference	Advanced: Any	81	77	Case-control	Blood	

Supplementary table 2. Factors involved in oxidative stress

	Reference	Up/down/ no difference	Type of AMD	Cases (n)	Controls (n)	Study design	Matrix	Comment
Malondialdehyde (MDA)	(Totan et al., 2001)	Up	Advanced: Neovascular	20	10	Case-control	Plasma	
	(Evereklioglu et al., 2003b)	Up	Any AMD	41	25	Cross-sectional	Plasma	Higher in advanced vs. early AMD Pos correlation NO, neg correlation SOD and GSHP
	(Yildirim et al., 2004)	Up	Advanced: Neovascular	30	60	Case-control	Plasma	
	(Baskol et al., 2006)	Up	Dry AMD	37	29	Case-control	Serum	Neg correlation PON1 activity
	(Ates et al., 2009)	Up	Advanced: Neovascular	40	40	Cross-sectional	Serum	Neg correlation PON1 activity
	(Totan et al., 2009)	Up	Advanced: Neovascular	47	25	Case-control	Serum	
	(Jia et al., 2011)	Up	Any AMD	56	34	Case-control	Serum	Higher in nAMD vs. early AMD Pos correlation SOD activity
	(Yildirim et al., 2011)	Up	Advanced: Neovascular	25	25	Case-control	Serum	
	(Shen et al., 2012)	Up/ Up/ Up	Early/ Advanced: GA/ Advanced: Neovascular	21/ 13/ 22	34	Case-control	Serum	Higher in nAMD vs. early AMD
	(Venza et al., 2012)	Up/ Up	Early AMD/ Advanced: Any	211/ 205	262	Case-control	Plasma, erythrocytes	
	(Park et al., 2014a)	Up	Advanced: Neovascular	42	84	Case-control	Plasma	Pos correlation ARMS2 risk genotype
Carboxyethyl-pyrrole (CEP) adducts	(Gu et al., 2009)	Up/ Up	Early/ Advanced: any	307/ 609	488	Case-control	Plasma	No significant differences between disease categories
	(Ni et al., 2009)	Up	Any AMD	54	32	Case-control	Plasma	Pos correlation with CML and pentosidine
	(Wang et al., 2014a)	Up	Any AMD	10	7	Case-control	Plasma	
Carboxyethyl-pyrrole (CEP) autoantibodies	(Gu et al., 2003)	Up	Any AMD	19	19	Case-control	Plasma	
	(Gu et al., 2009)	Up/ Up	Early/ Advanced: any	307/ 609	488	Case-control	Plasma	No significant difference between disease categories
	(Ni et al., 2009)	No difference	Any AMD	58	32	Case-control	Plasma	
N(6)-carboxymethyl-lysine (CML)	(Ni et al., 2009)	Up	Any AMD	58	32	Case-control	Plasma	Pos correlation with CEP adducts and pentosidine
	(Semba et al., 2014)	No difference/ No difference	Early AMD/ Advanced: Any	1025/ 276	3606	Population-based Cross-sectional	Serum	Age, Gene/Environment Susceptibility–Reykjavik Study
Protein Carbonyl groups (PCG)	(Totan et al., 2009)	Up	Advanced: Neovascular	47	25	Case-control	Serum	

	(Zafrilla et al., 2013)	Up	Advanced: Neovascular	163	170	Case-control	Serum	
Total Oxidation Status (TOS)	(Totan et al., 2009)	Up	Advanced: Neovascular	47	25	Case-control	Serum	Neg correlation TAC
	(Ugurlu et al., 2013)	Up	Advanced: Neovascular	22	23	Case-control	Serum	
Oxidized LDL (Ox-LDL)	(Ikeda et al., 2001)	Up	Advanced: Neovascular	72	140	Case-control	Plasma	
	(Klein et al., 2007b)	No difference	Early AMD	221	5666	Longitudinal	Serum	Multi-Ethnic Study of Atherosclerosis
	(Javadzadeh et al., 2010)	Up	Advanced: Neovascular	45	45	Case-control	Plasma	Pos correlation Hcy
	(Javadzadeh et al., 2012)	Up	Advanced: Neovascular	45	45	Case-control	Plasma	Note: this is the same cohort as described by (Javadzadeh et al., 2010)
Nitric Oxide (NO)	(Totan et al., 2001)	Down	Advanced: Neovascular	20	10	Case-control	Plasma	
	(Evereklioglu et al., 2003b)	Up	Any AMD	41	25	Cross-sectional	Plasma	Higher in Advanced: any vs. early AMD Neg correlation GSHP and SOD Pos correlation MDA
	(Tsai et al., 2006)	No difference/ No difference	Early AMD/ Advanced: neovascular	17/ 60	42	Case-control	Plasma	
Homocysteine (Hcy)	(Heuberger et al., 2002)	No difference/ No difference	Early AMD/ Advanced: Any	329/ 16	3182	Population-Based Cross-sectional	Serum	NHANES III
	(Axer-Siegel et al., 2004)	Up/ No difference	Advanced: Neovascular/ Dry AMD	59/ 58	56	Cross-sectional	Plasma	Higher in Advanced: Neovascular vs. Dry AMD
	(Vine et al., 2005)	Up	Any AMD	79	77	Case-control	Plasma	
	(Coral et al., 2006)	Up	Advanced: Neovascular	16	20	Case-control	Plasma	Neg correlation tSH and GSH
	(Kamburoglu et al., 2006)	Up/ Up	Advanced: Neovascular/ Dry AMD	30/ 30	30	Case-control	Plasma	
	(Seddon et al., 2006)	Up	Advanced: Any	222	184	Cross-sectional Case-control	Plasma	No statistical analysis were reported for early AMD (n=528)
	(Rochtchina et al., 2007)	Up	Advanced: Any	53	2910	Population-Based Cross-sectional	Serum	Blue Mountains Eye Study
	(Wu et al., 2007)	No difference/ No difference	Early AMD/ Advanced: Any	159/ 38	433	Population-Based Cross-sectional Case-control	Plasma	Blue Mountains Eye Study
	(Klein et al., 2008)	No difference/ No difference	Early AMD/ Advanced: Any	235/ 29	5623	Prospective Cohort	Serum	Multi-Ethnic Study of Atherosclerosis
	(Wang et al., 2008)	No difference	Any AMD	278	557	Population-Based Case-control	Serum	Blue Mountains Eye Study, Conflict between text and table!

	(Ates et al., 2009)	Up	Advanced: Neovascular	40	40	Cross-sectional	Serum	Neg correlation PON1 activity
	(Javadzadeh et al., 2010)	Up	Advanced: Neovascular	45	45	Case-control	Plasma	Pos correlation Ox-LDL
	(Javadzadeh et al., 2012)	Up	Advanced: Neovascular	45	45	Case-control	Plasma	Note: this is the same cohort as described by (Javadzadeh et al., 2010)
	(Ghosh et al., 2013)	Up/ No difference	Advanced: Neovascular/ Dry AMD	12/ 20	32	Case-control	Plasma	
	(Gopinath et al., 2013)	Up	Any AMD	219	1171	Cohort	Serum	Blue Mountains Eye Study, Subgroup analysis showed significant upregulation in early AMD, but not in advanced AMD
	(Obeid et al., 2013)	No difference/ No difference	Advanced: Neovascular/ Dry AMD	31/ 38	48	Case-control	Plasma	All cataract subjects Neg correlation esRAGE in Dry AMD
	(Christen et al., 2015)	No difference	Any AMD	452	27479	Prospective cohort	Plasma	Women only
	(Manresa et al., 2015)	Up	Advanced: Neovascular	73	80	Case-control	Vitreous, Plasma	Treatment naïve patients, treatment did not alter Hcy levels
Total Antioxidant Capacity (TAC)	(Eye-Disease-Case-Control-Study-Group, 1993)	Down	Advanced: Neovascular	391	578	Case-control	Serum	
	(Simonelli et al., 2002)	No difference/ No difference	Early AMD/ Advanced: Any	19/ 29	46	Case-control	Plasma	
	(Totan et al., 2009)	Down	Advanced: Neovascular	47	25	Case-control	Serum	Neg correlation TOS
	(Colak et al., 2012)	Down	Any AMD	84	84	Cross-sectional	Plasma	
	(Shen et al., 2012)	Down/ Down/ Down	Early/ Advanced: GA/ Advanced: Neovascular	21/ 13/ 22	34	Case-control	Serum	
	(Ugurlu et al., 2013)	No difference	Advanced: Neovascular	22	23	Case-control	Serum	
	(Zafrilla et al., 2013)	Down	Advanced: Neovascular	163	170	Case-control	Serum	
	(Plestina-Borjan et al., 2015)	Down	Any AMD	57	50	Case-control	Serum	
Thiol content (tSH)	(Coral et al., 2006)	Down	Advanced: Neovascular	16	20	Case-control	Plasma	Neg correlation Hcy
	(Javadzadeh et al., 2010)	Down	Advanced: Neovascular	45	45	Case-control	Plasma	
	(Ugurlu et al., 2013)	Down	Advanced: Neovascular	22	23	Case-control	Serum	
Glutathione (GSH)	(Samiec et al., 1998)	No difference	Any AMD	40	27	Case-control	Plasma	GSSG did not differ between AMD

								cases and age-matched controls
	(Delcourt et al., 1999b)	No difference	Advanced: Any	33	1895	Population-based	Erythrocytes	POLA, Same population as (Delcourt et al., 1999a)
	(Coral et al., 2006)	Down	Advanced: Neovascular	16	20	Case-control	Plasma	Neg correlation Hcy
	(Javadzadeh et al., 2010)	Down	Advanced: Neovascular	45	45	Case-control	Plasma	
	(Yildirim et al., 2011)	No difference	Advanced: Neovascular	25	25	Case-control	Serum	
	(Brantley et al., 2012)	No difference	Any AMD	69	67	Case-control	Plasma	
	(Qin et al., 2014)	No difference	Early	14	14	Case-control	Blood	GSSG (oxidized glutathione) higher in early AMD vs. controls
Glutathione Reductase (GSHR)	(Cohen et al., 1994)	Down	Any AMD	18	18	Case-control	Blood	
	(De La Paz et al., 1996)	No difference	Any AMD	54	12	Case-control	Erythrocytes	
	(Colak et al., 2012)	Down	Any AMD	84	84	Cross-sectional	Plasma	
	(Zafrilla et al., 2013)	Down	Advanced: Neovascular	163	170	Case-control	Serum	
Glutathione Peroxidase (GSHP)	(Prashar et al., 1993)	Down	Advanced: Neovascular	17	11	Case-control	Erythrocytes	
	(Cohen et al., 1994)	No difference	Any AMD	18	18	Case-control	Blood	
	(De La Paz et al., 1996)	No difference	Any AMD	54	12	Case-control	Erythrocytes	
	(Delcourt et al., 1999a)	No difference/ Up	Early/ Advanced: Any	642/ 38	1476	Population-Based Cross-sectional	Plasma	POLA
	(Evereklioglu et al., 2003b)	Down	Any AMD	41	25	Cross-sectional	Plasma, erythrocytes	Lower in advanced vs. early AMD Neg correlation NO and MDA
	(Yildirim et al., 2011)	No difference	Advanced: Neovascular	25	25	Case-control	Serum	
	(Colak et al., 2012)	No difference	Any AMD	84	84	Cross-sectional	Blood	
	(Venza et al., 2012)	Down/ Down	Early AMD/ Advanced: Any	211/ 205	262	Case-control	Plasma, erythrocytes	
	(Zafrilla et al., 2013)	No difference	Advanced: Neovascular	163	170	Case-control	Serum	
	(Plestina-Borjan et al., 2015)	Down	Any AMD	57	50	Case-control	Serum	
Carotenoids	(Eye-Disease-Case-Control-Study-Group, 1992)	Down	Advanced: Neovascular	414	606	Case-control	Serum	Sum of lutein/zeaxanthine, beta-carotene, a-carotene, cryptoxanthin and lycopene
	(Eye-Disease-Case-Control-Study-Group, 1993)	Down	Advanced: Neovascular	391	577	Case-control	Serum	
	(Simonelli et al., 2002)	No difference/ No difference	Early AMD/ Advanced: Any	19/ 29	46	Case-control	Plasma	Lower in advanced vs early AMD

	(Cardinault et al., 2005)	No difference	Any AMD	37	24	Case-control	Serum	Numbers reported in abstract differ from text and tables, fasting samples
Lutein	(Eye-Disease-Case-Control-Study-Group, 1993)	Down	Advanced: Neovascular	391	577	Case-control	Serum	Measured together with zeaxanthine
	(Sanders et al., 1993)	No difference	Any AMD	65	65	Case-control	Plasma	Correlation cholesterol
	(Mares-Perlman et al., 1995)	No difference	Any AMD	80	80	Case-control	Serum	
	(Simonelli et al., 2002)	No difference/ No difference	Early AMD/ Advanced: Any	19/ 29	46	Case-control	Serum	Measured together with zeaxanthine
	(Gale et al., 2003)	No difference	Any AMD	78	302	Case-control	Plasma	
	(Cardinault et al., 2005)	No difference	Any AMD	37	24	Case-control	Serum	Numbers reported in abstract differ from text and tables, fasting samples
	(Delcourt et al., 2006)	Down	Any AMD	41	599	Population-Based	Plasma	POLA
	(Michikawa et al., 2009)	No difference/ No difference	Early AMD/ Advanced: Any	32/ 8	682	Case-control	Serum	Measured together with zeaxanthine
	(Zhou et al., 2011)	No difference/ Down	Early AMD/ Advanced: Neovascular	92/ 82	89	Case-control	Serum	
Zeaxanthin	(Eye-Disease-Case-Control-Study-Group, 1993)	Down	Advanced: Neovascular	391	577	Case-control	Serum	Measured together with lutein
	(Mares-Perlman et al., 1995)	No difference	Any AMD	80	80	Case-control	Serum	
	(Simonelli et al., 2002)	No difference/ No difference	Early AMD/ Advanced: Any	19/ 29	46	Case-control	Serum	Measured together with lutein
	(Gale et al., 2003)	Down	Any AMD	78	302	Case-control	Plasma	
	(Cardinault et al., 2005)	No difference	Any AMD	37	24	Case-control	Serum	Numbers reported in abstract differ from text and tables, fasting samples
	(Delcourt et al., 2006)	Down	Any AMD	41	599	Population-Based	Plasma	POLA
	(Michikawa et al., 2009)	No difference/ No difference	Early AMD/ Advanced: Any	32/ 8	682	Case-control	Serum	Measured together with luteine
	(Zhou et al., 2011)	No difference/ Down	Early AMD/ Advanced: Neovascular	92/ 82	89	Case-control	Serum	
β-cryptoxanthin	(Eye-Disease-Case-Control-Study-Group, 1993)	Down	Advanced: Neovascular	391	577	Case-control	Serum	
	(Sanders et al., 1993)	No difference	Any AMD	65	65	Case-control	Plasma	Correlation cholesterol
	(Mares-Perlman et al.,	No difference	Any AMD	167	167	Case-control	Serum	

	1995)							
	(Simonelli et al., 2002)	No difference/ Down	Early AMD/ Advanced: Any	19/ 29	46	Case-control	Serum	Lower in advanced vs early AMD
	(Cardinault et al., 2005)	No difference	Any AMD	37	24	Case-control	Serum	Numbers reported in abstract differ from text and tables, fasting samples
	(Delcourt et al., 2006)	No difference	Any AMD	41	599	Population-Based	Plasma	POLA
	(Michikawa et al., 2009)	No difference/ Down	Early AMD/ Advanced: Any	32/ 8	682	Case-control	Serum	
	(Zhou et al., 2011)	No difference/ Down	Early AMD/ Advanced: Neovascular	92/ 82	89	Case-control	Serum	
α-carotene	(Eye-Disease-Case-Control-Study-Group, 1993)	Down	Advanced: Neovascular	391	577	Case-control	Serum	
	(Sanders et al., 1993)	No difference	Any AMD	65	65	Case-control	Plasma	
	(Mares-Perlman et al., 1995)	No difference	Any AMD	167	167	Case-control	Serum	
	(Cardinault et al., 2005)	No difference	Any AMD	37	24	Case-control	Serum	Numbers reported in abstract differ from text and tables, fasting samples
	(Delcourt et al., 2006)	No difference	Any AMD	41	599	Population-Based	Plasma	POLA
	(Michikawa et al., 2009)	No difference/ No difference	Early AMD/ Advanced: Any	32/ 8	682	Case-control	Serum	
	(Zhou et al., 2011)	Up/ Down	Early AMD/ Advanced: Neovascular	92/ 82	89	Case-control	Serum	
β-carotene	(Eye-Disease-Case-Control-Study-Group, 1993)	Down	Advanced: Neovascular	391	577	Case-control	Serum	
	(Sanders et al., 1993)	No difference	Any AMD	65	65	Case-control	Plasma	
	(West et al., 1994)	No difference	Any AMD	129	377	Case-control	Plasma	
	(Mares-Perlman et al., 1995)	No difference	Any AMD	167	167	Case-control	Serum	
	(Smith et al., 1997)	No difference/ No difference	Early AMD/ Advanced: Any	102/ 54	156	Case-control	Serum	Blue Mountains Eye Study, also no difference for pooled analysis of all AMD cases vs controls
	(Simonelli et al., 2002)	No difference/ No difference	Early AMD/ Advanced: Any	19/ 29	46	Case-control	Serum	
	(Cardinault et al., 2005)	No difference	Any AMD	37	24	Case-control	Serum	Numbers reported in abstract differ from text and tables, fasting samples
	(Delcourt et al., 2006)	No difference	Any AMD	41	599	Population-Based	Plasma	POLA

	(Michikawa et al., 2009)	No difference/ Down	Early AMD/ Advanced: Any	32/ 8	682	Case-control	Serum	
	(Zhou et al., 2011)	No difference/ Down	Early AMD/ Advanced: Neovascular	92/ 82	89	Case-control	Serum	
Lycopene	(Eye-Disease-Case-Control-Study-Group, 1993)	No difference	Advanced: Neovascular	391	577	Case-control	Serum	
	(Sanders et al., 1993)	No difference	Any AMD	65	65	Case-control	Plasma	Correlation cholesterol
	(Mares-Perlman et al., 1995)	No difference	Any AMD	167	167	Case-control	Serum	Logistic regression of high vs low levels revealed higher AMD risk for very low levels of lycopene
	(Simonelli et al., 2002)	Down/ Down	Early AMD/ Advanced: Any	19/ 29	46	Case-control	Serum	
	(Cardinault et al., 2005)	Down	Any AMD	37	24	Case-control	Serum	Numbers reported in abstract differ from text and tables
	(Delcourt et al., 2006)	No difference	Any AMD	41	599	Population-Based	Plasma	POLA
	(Michikawa et al., 2009)	No difference/ No difference	Early AMD/ Advanced: Any	32/ 8	682	Case-control	Serum	
	(Zhou et al., 2011)	No difference/ Down	Early AMD/ Advanced: Neovascular	92/ 82	89	Case-control	Serum	
Super Oxide Dismutase (SOD)	(Prashar et al., 1993)	Down	Advanced: Neovascular	17	11	Case-control	Erythrocytes	
	(Cohen et al., 1994)	No difference	Any AMD	18	18	Case-control	Erythrocytes	
	(De La Paz et al., 1996)	No difference	Any AMD	54	12	Case-control	Erythrocytes	
	(Delcourt et al., 1999a)	No difference/ No difference	Early/ Advanced: Any	642/ 38	1476	Population-Based Cross-sectional	Erythrocytes	POLA
	(Evereklioglu et al., 2003b)	Down	Any AMD	41	25	Cross-sectional	Plasma, erythrocytes	Lower in advanced vs. early AMD Neg correlation NO and MDA
	(Jia et al., 2011)	Up	Any AMD	56	34	Case-control	Serum	Activity levels, Pos correlation MDA
	(Yildirim et al., 2011)	Down	Advanced: Neovascular	25	25	Case-control	Serum	
	(Colak et al., 2012)	No difference	Any AMD	84	84	Cross-sectional	Blood hemolysate	
	(Shen et al., 2012)	No difference/ No difference/ Up	Early/ Advanced: GA/ Advanced: Neovascular	21/ 13/ 22	34	Case-control	Serum	Activity levels
	(Venza et al., 2012)	Down/ Down	Early AMD/ Advanced: Any	211/ 205	262	Case-control	Plasma, erythrocytes	Activity levels
	(Anand et al., 2013)	Up	Any AMD	115	61	Case-control	Serum	

	(Sharma et al., 2013b)	Up	Any AMD	73	33	Case-control	Serum	
	(Zafrilla et al., 2013)	Down	Advanced: Neovascular	163	170	Case-control	Serum	
	(Plestina-Borjan et al., 2015)	No difference	Any AMD	57	50	Case-control	Serum	
Paraoxonase 1 (PON1)	(Baskol et al., 2006)	Down	Dry AMD	37	29	Case-control	Serum	Activity level, Neg correlation MDA
	(Ates et al., 2009)	Down	Advanced: Neovascular	40	40	Cross-sectional	Serum	Activity level, Neg correlation MDA and Hcy
	(Javadzadeh et al., 2012)	No difference	Advanced: Neovascular	45	45	Case-control	Serum	Comparison of three PON1 phenotypes showed weak PON1 activity in nAMD compared to controls
	(Ugurlu et al., 2013)	Down	Advanced: Neovascular	22	23	Case-control	Serum	
Catalase	(Prashar et al., 1993)	Down	Advanced: Neovascular	17	11	Case-control	Erythrocytes	
	(De La Paz et al., 1996)	No difference	Any AMD	54	12	Case-control	Erythrocytes	
	(Everekliogl u et al., 2003b)	No difference	Any AMD	41	25	Cross-sectional	Erythrocytes	
	(Yildirim et al., 2004)	Down	Advanced: Neovascular	30	60	Case-control	Erythrocytes	Activity levels
	(Venza et al., 2012)	Down/ Down	Early AMD/ Advanced: Any	211/ 205	262	Case-control	Plasma, erythrocytes	Downregulation only in plasma, no significant difference in erythrocytes
	(Plestina-Borjan et al., 2015)	No difference	Any AMD	57	50	Case-control	Serum	

Supplementary table 3: Factors involved in immunity

	Reference	Up/down/ no difference	Type of AMD	Cases (n)	Controls (n)	Study design	Matrix	Comment
C3	(Scholl et al., 2008)	No difference	Any AMD	112	67	Case-control	Plasma	
	(Reynolds et al., 2009)	No difference/ No difference	Advanced: GA/ Advanced: Neovascular	58/ 62	60	Case-control	Plasma	
	(Silva et al., 2012)	No difference	Any AMD	119	152	Case-control	Plasma	
	(Smailhodzic et al., 2012)	No difference	Advanced: Neovascular	197	150	Case-control	Serum	EUGENDA
C3a	(Scholl et al., 2008)	Up	Any AMD	112	67	Case-control	Plasma	
	(Reynolds et al., 2009)	Up/ No difference	Advanced: GA/ Advanced: Neovascular	58/ 62	60	Case-control	Plasma	
C3d	(Scholl et al., 2008)	Up	Any AMD	112	67	Case-control	Plasma	
	(Hecker et al., 2010)	Up	Any AMD	125	149	Case-control	Plasma	
	(Smailhodzic et al., 2012)	Up	Advanced: Neovascular	197	150	Case-control	Serum	EUGENDA
C3a des Arg	(Sivaprasad et al., 2007)	Up	Early AMD/ Advanced: Neovascular	42/ 42	38	Case-control	Plasma	
	(Guymer et al., 2011)	No difference/ No difference/ No difference	Early AMD/ Advanced: GA/ Advanced: Any	51/ 19/ 33	54	Case-control	Urine	No correlation between serum and urinary C3a des Arg levels
C3d/C3	(Smailhodzic et al., 2012)	Up	Advanced: Neovascular	197	150	Case-control	Serum	EUGENDA
	(Ristau et al., 2014a)	Up/ Up	Any AMD/ Advanced: Any	864/ 495	1014	Case-control	Serum	EUGENDA, analysis for this marker only performed in subset of 1255 participants
	(Ristau et al., 2014b)	Up	Any AMD	1387	1268	Case-control	Serum	EUGENDA
C5a	(Scholl et al., 2008)	Up	Any AMD	112	67	Case-control	Plasma	
	(Reynolds et al., 2009)	Up/ No difference	Advanced: GA/ Advanced: Neovascular	58/ 62	60	Case-control	Plasma	Trend to upregulation in nAMD (P=0.09)
	(Hecker et al., 2010)	No difference	Any AMD	125	149	Case-control	Plasma	
	(Smailhodzic et al., 2012)	Up	Advanced: Neovascular	197	150	Case-control	Serum	EUGENDA
SC5b-9	(Scholl et al., 2008)	Up	Any AMD	112	67	Case-control	Plasma	
	(Reynolds et al., 2009)	No difference/ No difference	Advanced: GA/ Advanced: Neovascular	58/ 62	60	Case-control	Plasma	
	(Smailhodzic et al.,	No difference	Advanced: Neovascular	197	150	Case-control	Serum	EUGENDA

	2012)							
FH	(Hakobyan et al., 2008)	Up	Any AMD	53	75	Case-control	Plasma	Only in AMD patients with a heterozygous Y402H variant
	(Scholl et al., 2008)	No difference	Any AMD	112	67	Case-control	Plasma	
	(Reynolds et al., 2009)	Down/ No difference	Advanced: GA/ Advanced: Neovascular	58/ 62	60	Case-control	Plasma	Trend to downregulation in nAMD (P=0.06)
	(Silva et al., 2012)	No difference	Any AMD	119	152	Case-control	Plasma	
	(Smailhodzic et al., 2012)	No difference	Advanced: Neovascular	197	150	Case-control	Serum	EUGENDA
	(Ansari et al., 2013)	Down	Any AMD	382	201	Case-control	Plasma	
	(Sharma et al., 2013a)	Down/ Down	Dry AMD/ Advanced: Neovascular	31/ 84	61	Case-control	Serum	
	(Sharma et al., 2013b)	Down	Any AMD	73	33	Case-control	Serum	
	(Guymer et al., 2015)	No difference/ No difference/ No difference	Early AMD/ Advanced: GA/ Advanced: Neovascular	18/ 21/ 23	19	Cross-sectional Case-control	Serum	
FI	(Reynolds et al., 2009)	No difference/ No difference	Advanced: GA/ Advanced: Neovascular	58/ 62	60	Case-control	Plasma	
	(Silva et al., 2012)	Up	Any AMD	119	152	Case-control	Plasma	
	(Smailhodzic et al., 2012)	No difference	Advanced: Neovascular	197	150	Case-control	Serum	EUGENDA, Trend to upregulation (P=0.068)
	(van de Ven et al., 2013)	No difference	Any AMD	100	97	Case-control	Plasma	Significant downregulation in patients with Gly119Arg or Gly188Ala variant in the *CFI* gene
FB	(Scholl et al., 2008)	Up	Any AMD	112	67	Case-control	Plasma	
	(Reynolds et al., 2009)	No difference/ No difference	Advanced: GA/ Advanced: Neovascular	58/ 62	60	Case-control	Plasma	
	(Hecker et al., 2010)	Up	Any AMD	125	149	Case-control	Plasma	Not significant after correction and standardization
	(Silva et al., 2012)	No difference	Any AMD	119	152	Case-control	Plasma	Higher in women with AMD compared to men with AMD
	(Smailhodzic et al., 2012)	Up	Advanced: Neovascular	197	150	Case-control	Serum	EUGENDA
FD	(Scholl et al., 2008)	Up	Any AMD	112	67	Case-control	Plasma	
	(Reynolds et al., 2009)	No difference/ No difference	Advanced: GA/ Advanced: Neovascular	58/ 62	60	Case-control	Plasma	
	(Hecker et al., 2010)	Up	Any AMD	125	149	Case-control	Plasma	
	(Stanton et al., 2011)	Up	Any AMD	751	474	Case-control	Plasma	

	(Silva et al., 2012)	Down	Any AMD	119	152	Case-control	Plasma	
DAF/CD55	(Haas et al., 2011a)	No difference	Advanced: Neovascular	50	48	Case-control	Blood	
	(Singh et al., 2012)	No difference	Advanced: Neovascular	35	30	Case-control	Blood	
IL-1α	(Nassar et al., 2015)	Up	Advanced: Neovascular	30	15	Case-control	Serum	
	(Sakurada et al., 2015)	Up	Advanced: Neovascular	18	20	Case-control	Aqueous	Only in univariate analysis, not significant in multivariate analysis
IL-1β	(Mo et al., 2010)	No difference/ No difference/ No difference	Early AMD/ Advanced: GA/ Advanced: Neovascular	39/ 20/ 19	18	Case-control	Serum	
	(Nassar et al., 2015)	Up	Advanced: Neovascular	30	15	Case-control	Serum	
	(Zhao et al., 2015)	Up	Advanced: Neovascular	10	6	Prospective	Vitreous	
IL-2	(Klein et al., 2008)	No difference/ No difference	Early AMD/ Advanced: Any	235/ 29	5623	Prospective Cohort	Serum	Multi-Ethnic Study of Atherosclerosis
	(Nassar et al., 2015)	No difference	Advanced: Neovascular	30	15	Case-control	Serum	
	(Sakurada et al., 2015)	No difference	Advanced: Neovascular	18	20	Case-control	Aqueous	
IL-4	(Mo et al., 2010)	No difference/ No difference/ No difference	Early AMD/ Advanced: GA/ Advanced: Neovascular	39/ 20/ 19	18	Case-control	Serum	
	(Nassar et al., 2015)	Up	Advanced: Neovascular	30	15	Case-control	Serum	
	(Sakurada et al., 2015)	No difference	Advanced: Neovascular	18	20	Case-control	Aqueous	
IL-5	(Mo et al., 2010)	No difference/ No difference/ No difference	Early AMD/ Advanced: GA/ Advanced: Neovascular	39/ 20/ 19	18	Case-control	Serum	
	(Nassar et al., 2015)	Up	Advanced: Neovascular	30	15	Case-control	Serum	
IL-6	(Klein et al., 2005)	No difference	Any AMD	188	195	Nested Case-control	Serum	Beaver Dam Eye Study
	(Wu et al., 2007)	No difference/ No difference	Early AMD/ Advanced: Any	159/ 38	433	Population-Based Cross-sectional Case-control	Serum	Blue Mountains Eye Study
	(Klein et al., 2008)	No difference/ No difference	Early AMD/ Advanced: Any	235/ 29	5623	Prospective Cohort	Serum	Multi-Ethnic Study of Atherosclerosis, subgroup analysis in GA patients showed significant upregulation (n=18)
	(Wang et al., 2008)	No difference	Any AMD	188	393	Population-Based Case-control	Serum	Blue Mountains Eye Study, Conflict between text and table
	(Mo et al., 2010)	No difference/ No difference/ No difference	Early AMD/ Advanced: GA/ Advanced: Neovascular	39/ 20/ 19	18	Case-control	Serum	

	(Colak et al., 2012)	No difference	Any AMD	84	84	Cross-sectional	Serum	Subgroup analyses showed higher risk of AMD when IL-6>4.9 pg/ml (P=0.024)
	(Klein et al., 2014b)	Up	Early AMD	176	704	Longitudinal Population-Based Cohort	Serum	Beaver Dam Eye Study
	(Ambreen et al., 2015)	Up	Any AMD	90	100	Cross-sectional Case-control	Serum	IL-6 was sign higher in dry AMD vs nAMD
	(Aoki et al., 2015)	No difference	Any AMD	185	295	Cross-sectional	Serum	Hatoyama Cohort Study
	(Haas et al., 2015)	Up	Advanced: Neovascular	54	46	Case-control	Blood	
	(Nassar et al., 2015)	No difference	Advanced: Neovascular	30	15	Case-control	Serum	
	(Sakurada et al., 2015)	No difference	Advanced: Neovascular	18	20	Case-control	Aqueous	
IL-8	(Mo et al., 2010)	No difference/ No difference/ No difference	Early AMD/ Advanced: GA/ Advanced: Neovascular	39/ 20/ 19	18	Case-control	Serum	
	(Ambreen et al., 2015)	Up	Any AMD	90	100	Cross-sectional Case-control	Serum	IL-8 was sign higher in dry AMD vs nAMD
	(Nassar et al., 2015)	No difference	Advanced: Neovascular	30	15	Case-control	Serum	
	(Sakurada et al., 2015)	No difference	Advanced: Neovascular	18	20	Case-control	Aqueous	Trend to downregulation (P=0.09)
IL-10	(Mo et al., 2010)	No difference/ No difference/ No difference	Early AMD/ Advanced: GA/ Advanced: Neovascular	39/ 20/ 19	18	Case-control	Serum	
	(Nassar et al., 2015)	Up	Advanced: Neovascular	30	15	Case-control	Serum	
	(Sakurada et al., 2015)	No difference	Advanced: Neovascular	18	20	Case-control	Aqueous	
IL-12	(Nassar et al., 2015)	No difference	Advanced: Neovascular	30	15	Case-control	Serum	
	(Sakurada et al., 2015)	No difference	Advanced: Neovascular	18	20	Case-control	Aqueous	
IL-13	(Mo et al., 2010)	No difference/ No difference/ No difference	Early AMD/ Advanced: GA/ Advanced: Neovascular	39/ 20/ 19	18	Case-control	Serum	
	(Nassar et al., 2015)	Up	Advanced: Neovascular	30	15	Case-control	Serum	
	(Sakurada et al., 2015)	Down	Advanced: Neovascular	18	20	Case-control	Aqueous	P=0.05
IL-15	(Faber et al., 2015)	No difference/ No difference/ No difference	Early AMD/ Advanced: GA/ Advanced: Neovascular	30/ 16/ 90	74	Case-control	Plasma	
	(Nassar et al., 2015)	No difference	Advanced: Neovascular	30	15	Case-control	Serum	
	(Sakurada et al., 2015)	Up	Advanced: Neovascular	18	20	Case-control	Aqueous	Only in univariate analysis, not significant in multivariate analysis
IL-17	(Nassar et al., 2015)	Up	Advanced: Neovascular	30	15	Case-control	Serum	

	(Sakurada et al., 2015)	No difference	Advanced: Neovascular	18	20	Case-control	Aqueous	
IL-18	(Ijima et al., 2014)	Up/ No difference	Dry AMD/ Advanced: Neovascular	17/ 43	40	Case-control	Serum	
	(Faber et al., 2015)	No difference/ No difference/ No difference	Early AMD/ Advanced: GA/ Advanced: Neovascular	30/ 16/ 90	74	Case-control	Plasma	
CCL2	(Zhang et al., 2006)	Up	Any AMD	34	38	Case-control	Serum	
	(Mo et al., 2010)	No difference/ No difference/ No difference	Early AMD/ Advanced: GA/ Advanced: Neovascular	39/ 20/ 19	18	Case-control	Serum	
	(Guymer et al., 2011)	Up/ Up/ No difference	Early AMD/ Advanced: GA/ Advanced: Neovascular	51/ 19/ 33	54	Cross-sectional	Urine	No correlation between serum and urinary CCL2 levels
	(Anand et al., 2012)	Up	Any AMD	133	80	Case-control	Serum	
	(Grunin et al., 2012)	No difference	Advanced: Neovascular	30	27	Case-control	Serum	
	(Sharma et al., 2013b)	Up	Any AMD	73	33	Case-control	Serum	
	(Falk et al., 2014b)	No difference/ No difference	Early AMD/ Advanced: Neovascular	30/ 90	30	Case-control	Plasma	
	(Guymer et al., 2015)	No difference/ No difference/ No difference	Early AMD/ Advanced: GA/ Advanced: Neovascular	18/ 21/ 23	19	Cross-sectional Case-control	Serum	
	(Sakurada et al., 2015)	No difference	Advanced: Neovascular	18	20	Case-control	Aqueous	
CCR2 on Monocytes	(Zhang et al., 2006)	No difference	Any AMD	34	38	Case-control	Serum	
	(Anand et al., 2012)	Down	Any AMD	133	80	Case-control	Serum	
	(Grunin et al., 2012)	Up	Advanced: Neovascular	18	20	Case-control	Serum	
	(Falk et al., 2014b)	No difference/ No difference	Early AMD/ Advanced: Neovascular	30/ 90	30	Case-control	Plasma	
CCL11	(Mo et al., 2010)	Up/ Up/ No difference	Early AMD/ Advanced: GA/ Advanced: Neovascular	39/ 20/ 19	18	Case-control	Serum	
	(Falk et al., 2014a)	No difference	Advanced: Neovascular	83	114	Case-control	Plasma	Significant association with age
CCL24	(Sharma et al., 2012)	Up	Advanced: GA/ Advanced: Neovascular	38/ 95	80	Case-control	Serum	Higher CCL24 levels in nAMD vs. GA
	(Sharma et al., 2013b)	Up	Any AMD	73	33	Case-control	Serum	
CXCL10	(Mo et al., 2010)	Up/ Up/ Up	Early AMD/ Advanced: GA/ Advanced: Neovascular	39/ 20/ 19	18	Case-control	Serum	
	(Grunin et al., 2012)	No difference	Advanced: Neovascular	20	20	Case-control	Serum	

	(Falk et al., 2014c)	No difference/ No difference/ No difference	Early AMD/ Advanced: GA/ Advanced: Neovascular	30/ 12/ 89	31	Case-control	Plasma	
	(Sakurada et al., 2015)	Up	Advanced: Neovascular	18	20	Case-control	Aqueous	
CXCL12	(Machalinska et al., 2011a)	Down	Advanced: Neovascular	46	46	Case-control	Plasma	
	(Machalinska et al., 2011b)	Down	Advanced: Neovascular	29	38	Case-control	Plasma	
	(Scotti et al., 2014)	Up	Advanced: Neovascular	23	20	Case-control	Plasma	
	(Grierson et al., 2013)	No difference	Advanced: Neovascular	31	10	Case-control	Plasma	
CX3CR1	(Grunin et al., 2012)	No difference	Advanced: Neovascular	18	20	Case-control	Serum	
	(Falk et al., 2014b)	Up/ Up	Early AMD/ Advanced: Neovascular	30/ 90	30	Case-control	Plasma	Only in CD8+ cells
TNF-α	(Klein et al., 2005)	No difference	Any AMD	188	195	Nested Case-control	Serum	Beaver Dam Eye Study
	(Klein et al., 2008)	No difference/ No difference	Early AMD/ Advanced: Any	235/ 29	5623	Prospective Cohort	Serum	Multi-Ethnic Study of Atherosclerosis
	(Mo et al., 2010)	No difference/ No difference/ No difference	Early AMD/ Advanced: GA/ Advanced: Neovascular	39/ 20/ 19	18	Case-control	Serum	
	(Zehetner et al., 2014)	No difference	Advanced: Neovascular	30	12	Prospective Case-control	Plasma	
	(Guymer et al., 2015)	No difference/ No difference/ No difference	Early AMD/ Advanced: GA/ Advanced: Neovascular	18/ 21/ 23	19	Cross-sectional Case-control	Serum	
	(Haas et al., 2015)	No difference	Advanced: Neovascular	54	46	Case-control	Blood	
	(Nassar et al., 2015)	No difference	Advanced: Neovascular	30	15	Case-control	Serum	
TNF-α R2	(Klein et al., 2014b)	No difference	Early AMD	179	708	Longitudinal Population-Based Cohort	Serum	Beaver Dam Eye Study, Trend to upregulation (P=0.06)
	(Faber et al., 2015)	Up/ No difference/ Up	Early AMD/ Advanced: GA/ Advanced: Neovascular	30/ 16/ 90	74	Case-control	Plasma	
Interferon-gamma (IFN-γ)	(Mo et al., 2010)	No difference/ No difference/ No difference	Early AMD/ Advanced: GA/ Advanced: Neovascular	39/ 20/ 19	18	Case-control	Serum	
	(Faber et al., 2015)	No difference/ No difference/	Early AMD/ Advanced: GA/	30/ 16/	74	Case-control	Plasma	

		No difference	Advanced: Neovascular	90				
	(Nassar et al., 2015)	No difference	Advanced: Neovascular	30	15	Case-control	Serum	
CRP	(Klein et al., 2003a)	No difference	Early AMD	366	1995	Population-Based Cohort	Blood	Cardiovascular Health Study
	(Seddon et al., 2004)	No difference/ Up	Early AMD/ Advanced: Any	525/ 222	183	Case-control	Serum	
	(Dasch et al., 2005)	No difference/ Up	Early AMD/ Advanced: Any	422/ 270	181	Case-control	Serum	Muenster Aging and Retina Study, after correction this association was not significant
	(McGwin et al., 2005)	No difference	Any AMD	390	2365	Cross-sectional	Blood	Cardiovascular Health Study
	(Vine et al., 2005)	Up	Any AMD	79	77	Case-control	Serum	In model including both CRP and Hcy, CRP trends to upregulation (P=0.098)
	(Kikuchi et al., 2007)	Up	Advanced: Neovascular	176	262	Case-control	Serum	
	(Hogg et al., 2008)	No difference/ No difference	Dry AMD/ Advanced: Neovascular	97/ 195	115	Cross-sectional Case-control	Serum	
	(Roh et al., 2008)	No difference	Any AMD	235	9082	Case-control	Serum	Yonsei Eye Study
	(Ho et al., 2009)	No difference	Any AMD	164	1484	Population-Based Case-cohort	Blood	Rotterdam Study
	(Boey et al., 2010)	No difference/ No difference	Early AMD/ Advanced: Any	155/ 22	2923	Population-Based Cross-sectional	Serum	Singapore Malay Eye Study Highest vs lowest quartile CRP levels in non-diabetics were associated with advanced AMD
	(Robman et al., 2010)	No difference/ Up	Early AMD/ Advanced: Any	219/ 38	232	Case-control	Serum	
	(Seddon et al., 2010)	No difference/ Up	Early AMD/ Advanced: Any	175/ 69	209	Case-control	Serum	Age Related Eye Disease Ancillary Study
	(Subramani et al., 2010)	No difference	Any AMD	113	119	Case-control	Serum	Age-Related Macular Degeneration-Uric Acid Study
	(Colak et al., 2011)	Up	Any AMD	79	84	Cross-sectional	Serum	
	(Hong et al., 2011)	Up	Advanced: Any	-	-	Meta-analysis	Serum and plasma	Included 11 studies with a total of 41690 participants
	(Weiner et al., 2011)	Up	Any AMD	865	865	Case-control	Blood	
	(Colak et al., 2012)	No difference	Any AMD	84	84	Case-control	Serum	Subgroup analyses showed higher risk of AMD when CRP>3mg/l (P<0.05)
	(Silva et al., 2012)	No difference	Any AMD	119	152	Case-control	Plasma	
	(Cohn et al., 2013)	Up/ Up	Early AMD/ Advanced: Any	310/ 65	306	Cross-sectional	Blood	CHARM and ARMSS study, after adjustment only significant in

								advanced AMD
	(Singh et al., 2013a)	No difference	Advanced: Neovascular	62	44	Prospective Case-control	Serum	
	(Ulas et al., 2013)	Up	Advanced: Neovascular	142	141	Case-control	Serum	
	(Jonasson et al., 2014)	No difference	Any AMD	328	2540	Population-Based Cohort	Blood	Age Gene/Environment Susceptibility-Reykjavik Study
	(Semba et al., 2014)	Up	Early AMD/ Advanced: Any	1025/ 276	3606	Population-based cross-sectional	Serum	Age, Gene/Environment Susceptibility–Reykjavik Study
	(Ambreen et al., 2015)	Up	Any AMD	90	100	Cross-sectional Case-control	Serum	CRP was sign higher in wet vs dry
	(Guymer et al., 2015)	No difference/ No difference/ No difference	Early AMD/ Advanced: GA/ Advanced: Neovascular	18/ 21/ 23	19	Cross-sectional Case-control	Serum	
	(Min et al., 2015)	Up	Advanced: Neovascular	30	30	Case-control	Plasma	Positive correlation with PTx3
	(Sakurada et al., 2015)	Up	Advanced: Neovascular	18	20	Case-control	Aqueous	
	(Yip et al., 2015)	Up	Any AMD	673	4671	Prospective Cohort	Serum	EPIC Norfolk Eye Study
hsCRP	(Klein et al., 2005)	No difference	Any AMD	188	195	Nested Case-control	Serum	Beaver Dam Eye Study
	(Boekhoorn et al., 2007)	Up/ Up	Early AMD/ Advanced: Any	561/ 97	3946	Population-based Longitudinal	Serum	Rotterdam Study
	(Schaumberg et al., 2007)	Up	Any AMD	150	27537	Population-Based Longitudinal	Plasma	Women's Health Study, women only
	(Wu et al., 2007)	No difference/ No difference	Early AMD/ Advanced: Any	159/ 38	433	Population-Based Cross-sectional Case-control	Serum	Blue Mountains Eye Study
	(Klein et al., 2008)	No difference/ No difference	Early AMD/ Advanced: Any	235/ 29	5623	Prospective Cohort	Serum	Multi-Ethnic Study of Atherosclerosis, subgroup analysis in GA patients showed significant upregulation (n=18)
	(Wang et al., 2008)	No difference	Any AMD	188	393	Population-Based Case-control	Serum	Blue Mountains Eye Study, Conflict between text and table
	(Mitta et al., 2013)	Up	Any AMD	647	1480	Nested Case-control	Blood	Includes data from 5 studies
	(Klein et al., 2014b)	Up	Early AMD	178	697	Longitudinal Population-Based Cohort	Serum	Beaver Dam Eye Study
	(Aoki et al., 2015)	No difference	Any AMD	185	295	Cross-sectional	Serum	Hatoyama Cohort Study, Trend to

								upregulation in AMD (P=0.07)
	(Haas et al., 2015)	Up	Advanced: Neovascular	54	46	Case-control	Blood	
(soluble) ICAM	(Klein et al., 2005)	No difference	Any AMD	188	195	Nested Case-control	Serum	Beaver Dam Eye Study
	(Schaumberg et al., 2007)	Up	Any AMD	150	27537	Population-Based Longitudinal	Plasma	Women's Health Study, women only
	(Wu et al., 2007)	No difference/ No difference	Early AMD/ Advanced: Any	159/ 38	433	Population-Based Cross-sectional Case-control	Serum	Blue Mountains Eye Study, borderline significant upregulation in nAMD (P=0.0541) vs controls
	(Hogg et al., 2008)	No difference/ No difference	Dry AMD/ Advanced: Neovascular	97/ 195	115	Cross-sectional Case-control	Serum	
	(Wang et al., 2008)	No difference	Any AMD	188	393	Population-Based Case-control	Serum	Blue Mountains Eye Study
	(Klein et al., 2014b)	No difference	Early AMD	180	708	Longitudinal Population-Based Cohort	Serum	Beaver Dam Eye Study
	(Guymer et al., 2015)	No difference/ No difference/ No difference	Early AMD/ Advanced: GA/ Advanced: Neovascular	18/ 21/ 23	19	Cross-sectional Case-control	Serum	
(soluble) VCAM	(Hogg et al., 2008)	No difference/ No difference	Dry AMD/ Advanced: Neovascular	97/ 195	115	Cross-sectional Case-control	Serum	
	(Klein et al., 2014b)	Up	Early AMD	180	710	Longitudinal Population-Based Cohort	Serum	Beaver Dam Eye Study, upregulation found in fully adjusted model
	(Guymer et al., 2015)	No difference/ No difference/ No difference	Early AMD/ Advanced: GA/ Advanced: Neovascular	18/ 21/ 23	19	Cross-sectional Case-control	Serum	
WBC Count	(Blumenkranz et al., 1986)	Up	Advanced: Neovascular	26	23	Case-control	Blood	
	(Inhoffen and Nussgens, 1990)	No difference	Any AMD	35	35	Case-control	Blood	
	(Klein et al., 1993)	No difference/ Up/ No difference	Early AMD/ Advanced: Neovascular/ Advanced: GA	?	?	Population-Based	Blood	Beaver Dam Eye Study, total n=4771, case-control ratios not presented
	(Lip et al., 2001)	No difference	Any AMD	78	25	Cross-sectional	Blood	
	(Klein et al., 2003a)	No difference	Early AMD	366	1995	Population-Based Cohort	Blood	Cardiovascular Health Study
	(Klein et al., 2003c)	No difference/	Early AMD/	?	?	Population-Based	Blood	Beaver Dam Eye Study, total n=3674,

		No difference/ No difference	Advanced: GA/ Advanced: Neovascular			Cohort		case-control ratios not presented
	(Klein et al., 2005)	No difference	Any AMD	188	195	Nested Case-control	Serum	Beaver Dam Eye Study
	(Klein et al., 2007a)	No difference/ No difference/ Up	Early AMD/ Advanced: Neovascular/ Advanced: GA	866/ 39/ 14	3369	Observational	Blood	Women's Health Initiative Sight Examination, only women included
	(Shankar et al., 2007)	Up/ No difference	Early AMD/ Advanced: Any	249/ 63	3342	Population-based Cohort	Blood	Blue Mountains Eye Study
	(Wu et al., 2007)	No difference/ No difference	Early AMD/ Advanced: Any	159/ 38	433	Population-Based Cross-sectional Case-control	Blood	Blue Mountains Eye Study
	(Roh et al., 2008)	No difference	Any AMD	235	9082	Case-control	Serum	Yonsei Eye Study
	(Wang et al., 2008)	No difference	Any AMD	278	557	Population-Based Case-control	Serum	Blue Mountains Eye Study, Conflict between text and table
	(Klein et al., 2010)	No difference	Early AMD	96	2714	Cross-sectional	Blood	
	(Weiner et al., 2011)	Up	Any AMD	865	865	Cross-sectional Case-control	Blood	NHANESIII
	(Gopinath et al., 2013)	No difference	Any AMD	219	1171	Cohort	Blood	Blue Mountains Eye Study
	(Singh et al., 2013a)	No difference	Advanced: Neovascular	62	44	Prospective Case-control	Blood	
	(Cho et al., 2014)	Down/ No difference	Any AMD/ Advanced: Any	584/ 55	7315	Population-Based Cross-sectional	Serum	KNHANES, only significant in univariate analyses, not in multivariate
	(Klein et al., 2014b)	Up	Early AMD	181	711	Longitudinal Population-Based Cohort	Serum	Beaver Dam Eye Study, not significantly associated in fully adjusted model (P=0.16)
	(Joachim et al., 2015)	No difference	Early AMD	281	1036	Population-Based Cohort	Blood	Blue Mountains Eye Study, trend to downregulation (P=0.06)
Pentraxin 3 (PTX3)	(Juel et al., 2015)	No difference/ No difference/ No difference	Early AMD/ Advanced: GA/ Advanced: Neovascular	26/ 17/ 81	118	Case-control	Plasma	
	(Min et al., 2015)	Up	Advanced: Neovascular	30	30	Case-control	Plasma	Positive correlation with CRP
Anti-retinal autoantibodies (ARAs)	(Penfold et al., 1990)	Up	Any AMD	118	50	Case-control	Serum	Number of cases in text (128) does not match table (118). Most frequently observed staining pattern almost identical to anti-GFAP Ab.
	(Gurne et al., 1991)	Up	Any AMD	30	12	Case-control	Serum	

	(Patel et al., 2005)	Up/ Up	Early AMD/ Advanced: Neovascular	64/ 51	39	Case-control	Serum	
	(Cherepanoff et al., 2006)	Up	Early AMD	47	16	Longitudinal	Serum	Blue Mountains Eye Study, no association was found for progression to advanced AMD
	(Joachim et al., 2007)	Up & down	Advanced: Neovascular	39	101	Case-control	Serum	Different ARA profiles between cases and controls: upregulation of GFAP and α-enolase, downregulation of α-crystallin
	(Kubicka-Trzaska et al., 2012)	Up	Advanced: Neovascular	22	22	Longitudinal	Serum	ARAs decreased after anti-VEGF treatment
	(Morohoshi et al., 2012a)	Up	Any AMD	55	20	Case-control	Serum	Identification of 4 retinal antigens: Rbp3, ALDOC, PKM2, RLBP1
	(Adamus et al., 2014)	No difference/ No difference/ No difference	Early AMD/ Advanced: GA/ Advanced: Neovascular	41/ 28/ 33	26	Cross-sectional	Serum	Age-Related Eye Disease Study, specific ARAs associated with different severity stages
	(Kubicka-Trzaska et al., 2014)	Up	Advanced: Neovascular	98	50	Longitudinal	Serum	ARAs decreased after anti-VEGF treatment, correlated with lesion size and clinical outcomes anti-VEGF treatment
	(Iannaccone et al., 2015)	Up	Any AMD	131	231	Cross-sectional	Serum	Age-Related Maculopathy Ancillary Study, identification of HSPA8, HSPA9, CRYAA, ANXA5, S100A9
IgG anti-cardiolipin	(Ozkan et al., 2012)	Up/ Up	Dry AMD/ Advanced: Neovascular	19/ 23	25	Case-control	Plasma	
	(Morohoshi et al., 2012b)	No difference/ Up	Early AMD/ Advanced: Neovascular	35/ 20	20	Case-control	Serum	
Anti-Chlamydia pneumoniae	(Kalayoglu et al., 2003)	Up	Any AMD	25	18	Case-control	Serum	
	(Miller et al., 2004)	No difference/ No difference	Dry AMD/ Advanced: Neovascular	36/ 47	67	Case-control	Serum	
	(Klein et al., 2005)	No difference	Any AMD	188	195	Nested Case-control	Serum	Beaver Dam Eye Study
	(Robman et al., 2007)	No difference/ No difference	Early AMD/ Advanced: Any	159/ 38	433	Population-Based	Plasma	Blue Mountains Eye Study
	(Klein et al., 2008)	No difference/ No difference	Early AMD/ Advanced: Any	235/ 29	5623	Prospective Cohort	Serum	Multi-Ethnic Study of Atherosclerosis
Anti-cytomegalo-	(Miller et al., 2004)	No difference/	Dry AMD/	36/	67	Case-control	Serum	nAMD significantly higher than dry

virus (CMV)		Up	Advanced: Neovascular	47				AMD
	(Faber et al., 2013)	No difference	Any AMD	117	106	Case-control	Plasma	
Antibodies to H.pylori	(Miller et al., 2004)	No difference/ No difference	Dry AMD/ Advanced: Neovascular	36/ 47	67	Case-control	Serum	
	(Klein et al., 2008)	No difference/ No difference	Early AMD/ Advanced: Any	235/ 29	5623	Prospective Cohort	Serum	Multi-Ethnic Study of Atherosclerosis, NB: analysis in random subset of 1000 participants

Supplementary table 4: Factors involved in lipid metabolism

	Reference	Up/down/ no difference	Type of AMD	Cases (n)	Controls (n)	Study design	Matrix	Comment
(total) cholesterol	(Blumenkranz et al., 1986)	No difference	Advanced: Neovascular	26	23	Case-control	Serum	Fasting
	(Eye-Disease-Case-Control-Study-Group, 1992)	Up	Advanced: Neovascular	421	615	Case-control	Serum	
	(Tsang et al., 1992)	No difference	Any AMD	80	86	Case-control	Serum	Fasting
	(Klein et al., 1993)	No difference/ No difference/ No difference	Early AMD/ Advanced: Neovascular/ Advanced: GA	?	?	Population-Based	Serum	Beaver Dam Eye Study, down in female with early AMD, total n=4771, case-control ratios not presented
	(Sanders et al., 1993)	No difference	Any AMD	65	65	Case-control	Plasma	Nonfasting
	(Smith et al., 1998)	No difference/ No difference	Early AMD/ Advanced: Any	240/ 72	3342	Population-Based Cross-sectional	Serum	Blue Mountains Eye Study, fasting
	(Hyman et al., 2000)	No difference/ No difference	Dry AMD/ Advanced: Neovascular	227/ 182	235	Case-control	Serum	Fasting
	(Delcourt et al., 2001)	No difference/ No difference	Early AMD/ Advanced: Any	730/ 38	1372	Population-Based	Plasma	POLA, Fasting
	(Abalain et al., 2002)	No difference	Any AMD	84	62	Case-control	Serum	fasting
	(Klein et al., 2003a)	Down	Early AMD	366	1995	Population-Based Cohort	Plasma	Cardiovascular Health Study
	(Klein et al., 2003b)	No difference/ No difference/ No difference	Early AMD/ Advanced: Neovascular/ Advanced: GA	?	?	Population-Based Cohort	Serum	Beaver Dam Eye Study, trend to downregulation in nAMD (P=0.06), total n=2764, case-control ratios not presented
	(van Leeuwen et al., 2004)	No difference	Any AMD	414	4362	Population-Based Cohort	Serum	Rotterdam Study
	(Cardinault et al., 2005)	No difference	Any AMD	37	24	Case-control	Serum	Numbers reported in abstract differ from text and tables, fasting
	(Klein et al., 2005)	No difference	Any AMD	188	195	Nested Case-control	Serum	Beaver Dam Eye Study
	(McGwin et al., 2005)	Down	Any AMD	390	2365	Cross-sectional	Serum	Cardiovascular Health Study
	(Nowak et al., 2005)	Up	Dry AMD	60	45	Case-control	Serum	Fasting, only women included
	(Dashti et al., 2006)	No difference/	Early AMD/	58/	32	Cross-sectional	Plasma	

	No difference	Advanced: Any	39				
(Javadzadeh et al., 2007)	No difference	Advanced: Neovascular	60	60	Case-control	Serum	Fasting, only males included in study
(Klein et al., 2007b)	No difference	Early AMD	221	5666	Longitudinal	Serum	Multi-ethnic Study of Atherosclerosis, Fasting samples
(Tan et al., 2007)	No difference/ No difference	Early AMD/ Advanced: Any	?	?	Population-Based Cohort	Serum	Blue Mountains Eye Study, total n=2395, case-control ratios not presented
(Wu et al., 2007)	No difference/ No difference	Early AMD/ Advanced: Any	159/ 38	433	Population-Based Cross-sectional Case-control	Serum	Blue Mountains Eye Study, Fasting
(Cackett et al., 2008)	No difference/ No difference	Early AMD/ Advanced: Any	169/ 21	3075	Population-Based Cross-sectional	Serum	Singapore Malay Eye Study
(Hogg et al., 2008)	No difference/ Up	Dry AMD/ Advanced: Neovascular	97/ 195	115	Cross-sectional Case-control	Serum	Nonfasting
(Roh et al., 2008)	No difference	Any AMD	235	9082	Case-control	Serum	Yonsei Eye Study
(Ho et al., 2009)	No difference	Any AMD	164	1484	Population-Based case cohort	Blood	Rotterdam Study, nonfasting
(Boey et al., 2010)	No difference	Any AMD	177	2923	Population-Based Cross-sectional	Serum	Singapore Malay Eye Study
(Javadzadeh et al., 2010)	Up	Advanced: Neovascular	45	45	Case-control	Serum	Fasting
(Klein et al., 2010)	No difference	Early AMD	96	2714	Cross-sectional	Serum	
(Reynolds et al., 2010)	No difference/ No difference	Advanced: GA/ Advanced: Neovascular	123/ 195	140	Case-control	Serum	Fasting
(Rudnicka et al., 2010)	No difference	Advanced: Any	81	77	Case-control	Serum	Nonfasting
(Butt et al., 2011)	Down	Any AMD	347	639	Cross-sectional	Serum	Fasting
(Colak et al., 2011)	Up	Any AMD	79	84	Cross-sectional	Serum	Fasting
(Fauser et al., 2011)	No difference	Any AMD	792	521	Case-control	Serum	Nonfasting
(Weiner et al., 2011)	No difference	Any AMD	865	865	Cross-sectional Case-control	Blood	
(Fourgeux et al., 2012)	Up	Advanced: Any	128	71	Case-control	Plasma	Fasting
(Javadzadeh et al., 2012)	Up	Advanced: Neovascular	45	45	Case-control	Serum	Fasting, note: this is the same cohort as described by (Javadzadeh et al., 2010)
(Jonas et al., 2012)	No difference	Early AMD	215	4319	Population-Based Cross-sectional	Serum	Central India Eyes and Medical Study, postprandial, only 8 late cases

							thus not analyzed
(Wang et al., 2012)	No difference	Any AMD	161	2704	Population-Based	Serum	Beijing Eye Study, fasting
(Davari et al., 2013)	Up	Any AMD	32	32	Case-control	Serum	
(Munch et al., 2013)	No difference	Early AMD	251	644	Cross-sectional	Serum	Inter99 Study, fasting
(Ortak et al., 2013)	No difference	Any AMD	144	172	Case-control	Serum	Fasting
(Ulas et al., 2013)	Up	Advanced: Neovascular	142	141	Cross-sectional	Serum	Fasting
(Ambreen et al., 2014)	Up	Any AMD	90	100	Cross-sectional Case-control	Serum	
(Cho et al., 2014)	No difference/ No difference	Any AMD/ Advanced: Any	584/ 55	7315	Population-Based Cross-sectional	Serum	KNHANES
(Cougnard-Gregoire et al., 2014)	No difference/ No difference	Early AMD/ Advanced: Any	238/ 47	540	Population-Based	Plasma	ALIENOR, fasting
(Ersoy et al., 2014)	No difference	Advanced: Any	1147	1773	Case-control	Serum	Nonfasting
(Ghorbanihaghjo et al., 2014)	Up	Advanced: Neovascular	45	45	Cross-sectional	Serum	Fasting
(Jonasson et al., 2014)	No difference	Any AMD	328	2540	Population-Based Cohort	Plasma	Age Gene/Environment Susceptibility-Reykjavik Study
(Kim et al., 2014a)	No difference	Any AMD	312	4621	Population-Based Cross-sectional	Blood	KNHANES
(Kim et al., 2014b)	No difference/ No difference	Early AMD/ Advanced: Any	1163/ 115	15767	Population-Based Cross-sectional	Blood	KNHANES
(Klein et al., 2014a)	No difference/ No difference/ No difference	Early AMD/ Advanced: GA/ Advanced: Neovascular	?	?	Meta-analysis	Serum	Numbers are not clear from text, 3 population-based studies are included in meta-analysis (total n=6950)
(La et al., 2014)	No difference/ No difference	Early AMD/ Advanced: Any	1034/ 95	13223	Population-Based Cross-sectional	Blood	KNHANES, fasting
(Merle et al., 2014)	No difference	Advanced: Neovascular	290	144	RCT	Plasma	NAT2, fasting
(Park et al., 2014b)	No difference/ No difference	Early AMD/ Advanced: Any	958/ 88	12667	Population-based Cross-sectional	Serum	KNHANES
(Peiretti et al., 2014)	Down	Any AMD	136	38	Case-control	Plasma	
(Qin et al., 2014)	No difference	Early AMD	14	14	Case-control	Plasma	Fasting
(Semba et al., 2014)	No difference/ No difference	Early AMD/ Advanced: Any	1025/ 276	3606	Population-based cross-sectional	Serum	Age, Gene/Environment Susceptibility–Reykjavik Study
(Yang et al., 2014)	No difference	Early AMD	200	6377	Population-Based	Serum	Handan Eye Study, fasting
(Cezario et al., 2015)	No difference	Any AMD	30	30	Case-control	Serum	

	(Chaker et al., 2015)	No difference	Any AMD	805	4768	Population-Based Cohort	Blood	Rotterdam Study
	(Hwang et al., 2015)	No difference	Any AMD	312	4621	Population-Based Cross-sectional	Blood	KNHANES
	(Joachim et al., 2015)	No difference	Early AMD	281	1036	Population-Based Cohort	Serum	Blue Mountains Eye Study, Fasting
	(Min et al., 2015)	No difference	Advanced: Neovascular	30	30	Case-control	Plasma	
	(Paun et al., 2015)	No difference	Any AMD	1491	1579	Case-control	Serum	EUGENDA
	(Seshasai et al., 2015)	No difference	Any AMD	426	927	Population-Based Case-control	Serum	Singapore Indian Eye Study & Singapore Chinese Eye Study, nonfasting
	(Yip et al., 2015)	No difference	Any AMD	673	4671	Prospective Cohort	Serum	EPIC Norfolk Eye Study
Triglycerides (TG)	(Blumenkranz et al., 1986)	No difference	Advanced: Neovascular	26	23	Case-control	Serum	Fasting
	(Eye-Disease-Case-Control-Study-Group, 1992)	No difference	Advanced: Neovascular	421	615	Case-control	Serum	
	(Tsang et al., 1992)	No difference	Any AMD	80	86	Case-control	Serum	Fasting
	(Smith et al., 1998)	No difference/ No difference	Early AMD/ Advanced: Any	240/ 72	3342	Population-Based Cross-sectional	Serum	Blue Mountains Eye Study, fasting
	(Hyman et al., 2000)	No difference/ No difference	Dry AMD/ Advanced: Neovascular	227/ 182	235	Case-control	Serum	Fasting
	(Delcourt et al., 2001)	No difference/ No difference	Early AMD/ Advanced: Any	730/ 38	1372	Population-Based	Plasma	POLA, fasting
	(Abalain et al., 2002)	No difference	Any AMD	84	62	Case-control	Serum	Fasting
	(Klein et al., 2003a)	Down	Early AMD	366	1995	Population-Based Cohort	Plasma	Cardiovascular Health Study
	(Cardinault et al., 2005)	No difference	Any AMD	37	24	Case-control	Serum	Numbers reported in abstract differ from text and tables, fasting
	(Nowak et al., 2005)	Up	Dry AMD	60	45	Case-control	Serum	Fasting, only women included in this study
	(Javadzadeh et al., 2007)	No difference	Advanced: Neovascular	60	60	Case-control	Serum	Fasting, only male included in this study
	(Klein et al., 2007b)	No difference	Early AMD	221	5666	Longitudinal	Serum	Multi-Ethnic Study of Atherosclerosis, Fasting
	(Tan et al., 2007)	No difference/ No difference	Early AMD/ Advanced: Any	?	?	Population-Based Cohort	Serum	Blue Mountains Eye Study, total n=2395, case-control ratios not

							presented
(Cackett et al., 2008)	No difference/ No difference	Early AMD/ Advanced: Any	169/ 21	3075	Population-Based Cross-sectional	Serum	Singapore Malay Eye Study
(Hogg et al., 2008)	No difference/ No difference	Dry AMD/ Advanced: Neovascular	97/ 195	115	Cross-sectional Case-control	Serum	Nonfasting
(Roh et al., 2008)	Down	Any AMD	235	9082	Case-control	Serum	Yonsei Eye Study
(Boey et al., 2010)	Down	Any AMD	177	2923	Population-Based Cross-sectional	Serum	Singapore Malay Eye Study
(Javadzadeh et al., 2010)	No difference	Advanced: Neovascular	45	45	Case-control	Serum	Fasting
(Reynolds et al., 2010)	No difference/ No difference	Advanced: GA/ Advanced: Neovascular	123/ 195	140	Case-control	Serum	Fasting
(Colak et al., 2011)	No difference	Any AMD	79	84	Cross-sectional	Serum	Fasting
(Fauser et al., 2011)	No difference	Any AMD	780	521	Case-control	Serum	Nonfasting
(Weiner et al., 2011)	No difference	Any AMD	865	865	Cross-sectional Case-control	Not reported	
(Javadzadeh et al., 2012)	No difference	Advanced: Neovascular	45	45	Case-control	Serum	Fasting, note: this is the same cohort as described by (Javadzadeh et al., 2010)
(Wang et al., 2012)	No difference	Any AMD	161	2704	Population-Based	Serum	Beijing Eye Study, fasting
(You et al., 2012)	No difference	Early AMD	?	?	Population-Based	Blood	Beijing Eye Study, total n=3049, case-control ratios not presented
(Davari et al., 2013)	Up	Any AMD	32	32	Case-control	Serum	
(Munch et al., 2013)	Up	Early AMD	251	644	Cross-sectional	Serum	Inter99 Study, fasting, only significant higher levels in women
(Ortak et al., 2013)	No difference	Any AMD	144	172	Case-control	Serum	Fasting
(Ulas et al., 2013)	No difference	Advanced: Neovascular	142	141	Cross-sectional	Serum	Fasting
(Ambreen et al., 2014)	No difference	Any AMD	90	100	Cross-sectional Case-control	Serum	
(Cho et al., 2014)	No difference/ No difference	Any AMD/ Advanced: Any	584/ 55	7315	Population-Based Cross-sectional	Serum	KNHANES
(Cougnard-Gregoire et al., 2014)	No difference/ No difference	Early AMD/ Advanced: Any	238/ 47	540	Population-Based	Plasma	ALIENOR, Fasting
(Ersoy et al., 2014)	No difference	Advanced: Any	1147	1773	Case-control	Serum	Nonfasting
(Ghorbanihaghjo et al., 2014)	No difference	Advanced: Neovascular	45	45	Cross-sectional	Serum	Fasting

	(Kim et al., 2014a)	No difference	Any AMD	312	4621	Population-Based Cross-sectional	Blood	KNHANES
	(Kim et al., 2014b)	Down	Any AMD	1278	15767	Population-Based Cross-sectional	Blood	KNHANES
	(La et al., 2014)	No difference/ No difference	Early AMD/ Advanced: Any	1034/ 95	13223	Population-Based Cross-sectional	Blood	KNHANES, fasting
	(Merle et al., 2014)	Down	Advanced: Neovascular	290	144	RCT	Plasma	NAT2, fasting
	(Park et al., 2014b)	No difference/ No difference	Early AMD/ Advanced: Any	958/ 88	12667	Population-based Cross-sectional	Serum	KNHANES
	(Qin et al., 2014)	No difference	Early AMD	14	14	Case-control	Plasma	Fasting
	(Semba et al., 2014)	Down/ Down	Early AMD/ Advanced: Any	1025/ 276	3606	Population-based cross-sectional	Serum	Age, Gene/Environment Susceptibility–Reykjavik Study
	(Yang et al., 2014)	Down	Early AMD	200	6377	Population-Based	Serum	Handan Eye Study, fasting
	(Cezario et al., 2015)	No difference	Any AMD	30	30	Case-control	Serum	
	(Hwang et al., 2015)	No difference	Any AMD	312	4621	Population-Based Cross-sectional	Blood	KNHANES
	(Joachim et al., 2015)	No difference	Early AMD	281	1036	Population-Based Cohort	Serum	Blue Mountains Eye Study, fasting
	(Paun et al., 2015)	Down	Any AMD	1491	1579	Case-control	Serum	EUGENDA
	(Yip et al., 2015)	No difference	Any AMD	673	4671	Prospective Cohort	Serum	EPIC Norfolk Eye Study
Phospholipids	(Sanders et al., 1993)	No difference	Any AMD	65	65	Case-control	Plasma	Nonfasting
	(Abalain et al., 2002)	No difference	Any AMD	84	62	Case-control	Serum	Fasting
	(Cardinault et al., 2005)	No difference	Any AMD	37	24	Case-control	Serum	Numbers reported in abstract differ from text and tables, fasting
LDL-C	(Blumenkranz et al., 1986)	No difference	Advanced: Neovascular	26	23	Case-control	Serum	Fasting
	(Hyman et al., 2000)	No difference/ No difference	Dry AMD/ Advanced: Neovascular	227/ 182	235	Case-control	Serum	Fasting
	(Abalain et al., 2002)	No difference	Any AMD	84	62	Case-control	Serum	Fasting
	(Klein et al., 2003a)	Down	Early AMD	366	1995	Population-Based Cohort	Plasma	Cardiovascular Health Study
	(McGwin et al., 2005)	No difference	Any AMD	390	2365	Cross-sectional	Serum	Cardiovascular Health Study, Trend to downregulation (P=0.0679)
	(Nowak et al., 2005)	Up	Dry AMD	60	45	Case-control	Serum	Fasting, only women included in study
	(Javadzadeh et al.,	Up	Advanced: Neovascular	60	60	Case-control	Serum	Fasting, only males included in study

2007)							
(Klein et al., 2007b)	No difference	Early AMD	221	5666	Longitudinal	Serum	Multi-ethnic Study of Atherosclerosis, fasting
(Tan et al., 2007)	No difference/ No difference	Early AMD/ Advanced: Any	?	?	Population-Based Cohort	Serum	Blue Mountains Eye Study, total n=2395, case-control ratios not presented
(Cackett et al., 2008)	No difference/ No difference	Early AMD/ Advanced: Any	169/ 21	3075	Population-Based Cross-sectional	Serum	Singapore Malay Eye Study
(Roh et al., 2008)	No difference	Any AMD	235	9082	Case-control	Serum	Yonsei Eye Study
(Boey et al., 2010)	No difference	Any AMD	177	2923	Population-Based Cross-sectional	Serum	Singapore Malay Eye Study
(Javadzadeh et al., 2010)	Up	Advanced: Neovascular	45	45	Case-control	Serum	Fasting
(Reynolds et al., 2010)	No difference/ Up	Advanced: GA/ Advanced: Neovascular	123/ 195	140	Case-control	Serum	Fasting
(Colak et al., 2011)	Up	Any AMD	79	84	Cross-sectional	Serum	Fasting sample
(Weiner et al., 2011)	No difference	Any AMD	865	865	Cross-sectional Case-control	Blood	
(Javadzadeh et al., 2012)	Up	Advanced: Neovascular	45	45	Case-control	Serum	Fasting, note: this is the same cohort as described by (Javadzadeh et al., 2010)
(Wang et al., 2012)	No difference	Any AMD	161	2704	Population-Based	Serum	Beijing Eye Study, fasting
(You et al., 2012)	No difference	Early AMD	?	?	Population-Based	Blood	Beijing Eye Study, total n=3049, case-control ratios not presented
(Davari et al., 2013)	Up	Any AMD	32	32	Case-control	Serum	
(Munch et al., 2013)	No difference	Early AMD	251	644	Cross-sectional	Serum	Inter99 Study, fasting
(Ortak et al., 2013)	No difference	Any AMD	144	172	Case-control	Serum	Fasting
(Ulas et al., 2013)	Up	Advanced: Neovascular	142	141	Cross-sectional	Serum	Fasting
(Ambreen et al., 2014)	No difference	Any AMD	90	100	Cross-sectional Case-control	Serum	
(Cho et al., 2014)	No difference/ No difference	Any AMD/ Advanced: Any	584/ 55	7315	Population-Based Cross-sectional	Serum	KNHANES
(Cougnard-Gregoire et al., 2014)	No difference/ No difference	Early AMD/ Advanced: Any	238/ 47	540	Population-Based	Plasma	ALIENOR, Fasting
(Ghorbanihaghjo et al., 2014)	Up	Advanced: Neovascular	45	45	Cross-sectional	Serum	Fasting

	(Merle et al., 2014)	No difference	Advanced: Neovascular	290	144	RCT	Plasma	NAT2, fasting
	(Qin et al., 2014)	Up	Early AMD	14	14	Case-control	Plasma	Fasting
	(Semba et al., 2014)	No difference	Early AMD/ Advanced: Any	1025/ 276	3606	Population-based cross-sectional	Serum	Age, Gene/Environment Susceptibility–Reykjavik Study
	(Yang et al., 2014)	No difference	Early AMD	200	6377	Population-Based	Serum	Handan Eye Study, fasting
	(Cezario et al., 2015)	No difference	Any AMD	30	30	Case-control	Serum	
	(Paun et al., 2015)	No difference	Any AMD	1491	1579	Case-control	Serum	EUGENDA
	(Yip et al., 2015)	No difference	Any AMD	673	4671	Prospective Cohort	Serum	EPIC Norfolk Eye Study
nonHDL-C	(Colak et al., 2011)	Up	Any AMD	79	84	Cross-sectional	Serum	Fasting
	(Klein et al., 2014a)	No difference/ No difference/ No difference	Early AMD/ Advanced: GA/ Advanced: Neovascular	?	?	Meta-analysis	Serum	Numbers are not clear from text, 3 population-based studies are included in meta-analysis (total n=6950)
	(Paun et al., 2015)	No difference	Any AMD	1491	1579	Case-control	Serum	EUGENDA
HDL-C	(Blumenkranz et al., 1986)	No difference	Advanced: Neovascular	26	23	Case-control	Serum	Fasting
	(Eye-Disease-Case-Control-Study-Group, 1992)	No difference	Advanced: Neovascular	421	615	Case-control	Serum	
	(Klein et al., 1993)	Up/ No difference/ No difference	Early AMD/ Advanced: Neovascular/ Advanced: GA	?	?	Population-Based	Serum	Beaver Dam Eye Study, association with HDL-D only found in male participants, total n=4771, case-control ratios not presented
	(Smith et al., 1998)	No difference/ No difference	Early AMD/ Advanced: Any	240/ 72	3342	Population-Based Cross-sectional	Serum	Blue Mountains Eye Study, fasting
	(Hyman et al., 2000)	No difference/ Up	Dry AMD/ Advanced: Neovascular	227/ 182	235	Case-control	Serum	Fasting
	(Delcourt et al., 2001)	Up/ No difference	Early AMD/ Advanced: Any	730/ 38	1372	Population-Based	Plasma	POLA, higher levels of HDL-C in patients with soft drusen
	(Abalain et al., 2002)	No difference	Any AMD	84	62	Case-control	Serum	Fasting
	(Klein et al., 2003b)	No difference/ No difference/ Up	Early AMD/ Advanced: Neovascular/ Advanced: GA	?	?	Population-Based Cohort	Serum	Beaver Dam Eye Study, Total n=2764, case-control ratios not presented
	(van Leeuwen et al., 2004)	Up	Any AMD	414	4352	Population-Based Cohort	Serum	Rotterdam Study
	(Klein et al., 2005)	No difference	Any AMD	188	195	Nested	Serum	Beaver Dam Eye Study

					Case-control		
(McGwin et al., 2005)	No difference	Any AMD	390	2365	Cross-sectional	Serum	Cardiovascular Health Study
(Nowak et al., 2005)	Down	Dry AMD	60	45	Case-control	Serum	Fasting, only women included
(Javadzadeh et al., 2007)	No difference	Advanced: Neovascular	60	60	Case-control	Serum	Fasting, only males included in study
(Klein et al., 2007b)	Up	Early AMD	221	5666	Longitudinal	Serum	Multi-Ethnic Study of Atherosclerosis, fasting, higher HDL-C in AMD only borderline significant in multivariate analysis (0.05<P<0.10)
(Tan et al., 2007)	No difference/ Down	Early AMD/ Advanced: Any	?	?	Population-Based Cohort	Serum	Blue Mountains Eye Study, total n=2395, case-control ratios not presented
(Wu et al., 2007)	No difference/ No difference	Early AMD/ Advanced: Any	159/ 38	433	Population-Based Cross-sectional Case-control	Serum	Blue Mountains Eye Study, Fasting
(Cackett et al., 2008)	No difference/ No difference	Early AMD/ Advanced: Any	169/ 21	3075	Population-Based Cross-sectional	Serum	Singapore Malay Eye Study
(Hogg et al., 2008)	No difference/ No difference	Dry AMD/ Advanced: Neovascular	97/ 195	115	Cross-sectional Case-control	Serum	Nonfasting
(Roh et al., 2008)	No difference	Any AMD	235	9082	Case-control	Serum	Yonsei Eye Study
(Ho et al., 2009)	Up	Any AMD	164	1484	Population-Based case cohort	Blood	Rotterdam Study, nonfasting
(Boey et al., 2010)	No difference	Any AMD	177	2923	Population-Based Cross-sectional	Serum	Singapore Malay Eye Study
(Javadzadeh et al., 2010)	No difference	Advanced: Neovascular	45	45	Case-control	Serum	Fasting
(Klein et al., 2010)	Down	Early AMD	96	2714	Cross-sectional	Serum	
(Reynolds et al., 2010)	No difference/ Down	Advanced: GA/ Advanced: Neovascular	123/ 195	140	Case-control	Serum	Fasting
(Butt et al., 2011)	Up	Any AMD	347	639	Cross-sectional	Serum	Fasting
(Colak et al., 2011)	No difference	Any AMD	79	84	Cross-sectional	Serum	Fasting
(Fauser et al., 2011)	No difference	Any AMD	805	521	Case-control	Serum	Nonfasting
(Weiner et al., 2011)	Up	Any AMD	865	865	Cross-sectional Case-control	Blood	Only significant in Any AMD, not significantly associated with late AMD
(Javadzadeh et al., 2012)	No difference	Advanced: Neovascular	45	45	Case-control	Serum	Fasting, note: this is the same cohort as described by (Javadzadeh et al.,

							2010)
(Jonas et al., 2012)	No difference	Early AMD	215	4319	Population-Based Cross-sectional	Serum	Central India Eyes and Medical Study, Postprandial, only 8 late cases thus not analyzed
(Wang et al., 2012)	No difference	Any AMD	161	2704	Population-Based	Serum	Beijing Eye Study, fasting
(You et al., 2012)	No difference	Early AMD	?	?	Population-Based	Blood	Beijing Eye Study, total n=3049, case-control ratios not presented
(Davari et al., 2013)	No difference	Any AMD	32	32	Case-control	Serum	
(Munch et al., 2013)	No difference	Early AMD	251	644	Cross-sectional	Serum	Inter99 Study, fasting
(Ortak et al., 2013)	No difference	Any AMD	144	172	Case-control	Serum	Fasting
(Ulas et al., 2013)	No difference	Advanced: Neovascular	142	141	Cross-sectional	Serum	Trend to downregulation in nAMD (P=0.081), fasting
(Ambreen et al., 2014)	No difference	Any AMD	90	100	Cross-sectional Case-control	Serum	
(Cho et al., 2014)	Up/ No difference	Any AMD/ Advanced: Any	584/ 55	7315	Population-Based Cross-sectional	Serum	KNHANES
(Cougnard-Gregoire et al., 2014)	Up/ Up	Early AMD/ Advanced: Any	238/ 47	540	Population-Based	Plasma	ALIENOR, fasting
(Ersoy et al., 2014)	No difference	Advanced: Any	1147	1773	Case-control	Serum	Nonfasting
(Ghorbanihaghjo et al., 2014)	No difference	Advanced: Neovascular	45	45	Cross-sectional	Serum	Fasting
(Jonasson et al., 2014)	Up	Any AMD	328	2540	Population-Based Cohort	Plasma	Age Gene/Environment Susceptibility-Reykjavik Study
(Klein et al., 2014a)	No difference/ No difference/ No difference	Early AMD/ Advanced: GA/ Advanced: Neovascular	?	?	Meta-analysis	Serum	Numbers are not clear from text, 3 population-based studies are included in meta-analysis (total n=6950)
(Merle et al., 2014)	No difference	Advanced: Neovascular	290	144	RCT	Plasma	NAT2, fasting
(Park et al., 2014b)	No difference/ No difference	Early AMD/ Advanced: Any	626/ 58	9189	Population-based Cross-sectional	Serum	KNHANES, Trend to upregulation in early AMD (P=0.057)
(Peiretti et al., 2014)	No difference	Any AMD	136	38	Case-control	Plasma	
(Qin et al., 2014)	No difference	Early AMD	14	14	Case-control	Plasma	Fasting
(Semba et al., 2014)	No difference/ Up	Early AMD/ Advanced: Any	1025/ 276	3606	Population-based cross-sectional	Serum	Age, Gene/Environment Susceptibility–Reykjavik Study
(Yang et al., 2014)	No difference	Early AMD	200	6377	Population-Based	Serum	Handan Eye Study, fasting
(Aoki et al., 2015)	Up	Any AMD	185	295	Cross-sectional	Serum	Hatoyama Cohort Study

	(Cezario et al., 2015)	Down?	Any AMD	30	30	Case-control	Serum	Conflict between abstract and text
	(Joachim et al., 2015)	No difference	Early AMD	281	1036	Population-Based Cohort	Serum	Blue Mountains Eye Study, fasting
	(Paun et al., 2015)	Up	Any AMD	1491	1579	Case-control	Serum	EUGENDA
	(Seshasai et al., 2015)	No difference	Any AMD	426	927	Population-Based Case-control	Serum	Singapore Indian Eye Study & Singapore Chinese Eye Study, nonfasting, trend to upregulation (P=0.08)
	(Yip et al., 2015)	Up	Any AMD	673	4671	Prospective Cohort	Serum	EPIC Norfolk Eye Study
lp(a)	(Abalain et al., 2002)	No difference	Any AMD	84	62	Case-control	Serum	Fasting
	(Klein et al., 2003a)	No difference	Early AMD	366	1995	Population-Based Cohort	Plasma	Cardiovascular Health Study
	(Nowak et al., 2005)	No difference	Dry AMD	60	45	Case-control	Serum	Fasting, only women included, trend to upregulation (P=0.065)
	(Colak et al., 2011)	No difference	Any AMD	79	84	Cross-sectional	Serum	Fasting
	(Fauser et al., 2011)	No difference	Any AMD	691	397	Case-control	Serum	Nonfasting
	(Ersoy et al., 2014)	No difference	Advanced: Any	1147	1773	Case-control	Serum	Nonfasting
apoA1	(Delcourt et al., 2001)	Up/ No difference	Early AMD/ Advanced: Any	730/ 38	1372	Population-Based	Plasma	POLA, Higher levels of ApoA1 in patients with soft drusen
	(Abalain et al., 2002)	No difference	Any AMD	84	62	Case-control	Serum	Fasting
	(Nowak et al., 2005)	Down	Dry AMD	60	45	Case-control	Serum	Fasting, only women included
	(Dashti et al., 2006)	No difference/ No difference	Early AMD/ Advanced: Any	58/ 39	32	Cross-sectional	Plasma	
	(Colak et al., 2011)	No difference	Any AMD	79	84	Cross-sectional	Serum	Fasting
	(Fauser et al., 2011)	No difference	Any AMD	690	398	Case-control	Serum	EUGENDA, nonfasting, in paper ApoA2 but rectification to ApoA1
	(Ersoy et al., 2014)	No difference	Advanced: Any	1147	1773	Case-control	Serum	EUGENDA, nonfasting, in paper ApoA2 but rectification to ApoA1
	(Paun et al., 2015)	Up	Any AMD	1491	1579	Case-control	Serum	EUGENDA
apoB	(Delcourt et al., 2001)	No difference/ No difference	Early AMD/ Advanced: Any	730/ 38	1372	Population-Based	Plasma	POLA, fasting
	(Abalain et al., 2002)	No difference	Any AMD	84	62	Case-control	Serum	Fasting
	(Nowak et al., 2005)	Up	Dry AMD	60	45	Case-control	Serum	Fasting, only women included
	(Dashti et al., 2006)	No difference/ No difference	Early AMD/ Advanced: Any	58/ 39	32	Cross-sectional	Plasma	

	(Colak et al., 2011)	No difference	Any AMD	79	84	Cross-sectional	Serum	Fasting
	(Fauser et al., 2011)	Up	Any AMD	689	398	Case-control	Serum	Nonfasting
	(Ersoy et al., 2014)	No difference	Advanced: Any	1147	1773	Case-control	Serum	Nonfasting
	(Paun et al., 2015)	No difference	Any AMD	1491	1579	Case-control	Serum	EUGENDA
apoE	(Abalain et al., 2002)	Up	Any AMD	84	62	Case-control	Serum	Subgroup analyses showed significantly higher apoE levels in advanced AMD vs early AMD.
	(Klein et al., 2003a)	No difference	Early AMD	366	1995	Population-based Cohort	Plasma	Cardiovascular Health Study
Docosahexaenoic acid (DHA)	(Sanders et al., 1993)	No difference	Any AMD	65	65	Case-control	Plasma, erythrocytes	
	(Ouchi et al., 2002)	Up	Any AMD	11	10	Case-control	Plasma, erythrocytes	Only significant in erythrocytes
	(Kabasawa et al., 2011)	No difference/ No difference	Dry AMD/ Advanced: Neovascular	31/ 166	143	Case-control	Serum	Fasting
	(Merle et al., 2013)	Down	Advanced: Any	64	541	Population-Based	Plasma	ALIENOR, fasting, controls are defined as without late AMD
	(Merle et al., 2014)	Down	Advanced: Neovascular	290	144	RCT	RBCM, Serum	NAT2, fasting, only in RBCM
	(Orban et al., 2015)	Down	Advanced: Neovascular	21	22	Case-control	Serum	
Eicosapentaenoic acid (EPA)	(Sanders et al., 1993)	No difference	Any AMD	65	65	Case-control	Plasma, erythrocytes	
	(Ouchi et al., 2002)	No difference	Any AMD	11	10	Case-control	Plasma, erythrocytes	Trend to upregulation in erythrocytes (P=0.07)
	(Kabasawa et al., 2011)	No difference/ No difference	Dry AMD/ Advanced: Neovascular	31/ 166	143	Case-control	Serum	Trend to upregulation in nAMD (P=0.053)
	(Merle et al., 2013)	No difference	Advanced: Any	64	541	Population-Based	Plasma	ALIENOR, controls are defined as without late AMD, trend to downregulation (P=0.07), only significant in Advanced: GA (P=0.007)
	(Merle et al., 2014)	Down	Advanced: Neovascular	290	144	RCT	RBCM, Serum	NAT2
α-Linolenic acid (ALA)	(Kabasawa et al., 2011)	No difference/ No difference	Dry AMD/ Advanced: Neovascular	31/ 166	143	Case-control	Serum	
	(Merle et al., 2013)	Down	Advanced: Any	64	541	Population-Based	Plasma	ALIENOR, controls are defined as without late AMD
Docosapentaenoic	(Sanders et al., 1993)	No difference	Any AMD	65	65	Case-control	Plasma,	

acid (DPA)							erythrocytes	
	(Kabasawa et al., 2011)	No difference/ No difference	Dry AMD/ Advanced: Neovascular	31/ 166	143	Case-control	Serum	
	(Merle et al., 2013)	No difference	Advanced: Any	64	541	Population-Based	Plasma	ALIENOR, Trend to downregulation (P=0.05)
Arachidonic acid (AA)	(Sanders et al., 1993)	No difference	Any AMD	65	65	Case-control	Plasma, erythrocytes	
	(Ouchi et al., 2002)	Up	Any AMD	11	10	Case-control	Plasma, erythrocytes	Only significant in erythrocytes
	(Kabasawa et al., 2011)	No difference/ No difference	Dry AMD/ Advanced: Neovascular	31/ 166	143	Case-control	Serum	Fasting
	(Orban et al., 2015)	Up	Advanced: Neovascular	22	21	Case-control	Serum	Numbers are conflicting in text and figure
Linoleic acid (LA)	(Sanders et al., 1993)	No difference	Any AMD	65	65	Case-control	Plasma, erythrocytes	
	(Ouchi et al., 2002)	Down	Any AMD	11	10	Case-control	Plasma, erythrocytes	Only significant in erythrocytes
	(Kabasawa et al., 2011)	No difference/ No difference	Dry AMD/ Advanced: Neovascular	31/ 166	143	Case-control	Serum	Fasting
Oleic acid (OA)	(Ouchi et al., 2002)	Down	Any AMD	11	10	Case-control	Plasma, erythrocytes	Only significant in erythrocytes
	(Kabasawa et al., 2011)	No difference/ No difference	Dry AMD/ Advanced: Neovascular	31/ 166	143	Case-control	Serum	Fasting
Palmitic acid (PA)	(Ouchi et al., 2002)	Down	Any AMD	11	10	Case-control	Plasma, erythrocytes	Only significant in erythrocytes
	(Kabasawa et al., 2011)	No difference/ No difference	Dry AMD/ Advanced: Neovascular	31/ 166	143	Case-control	Serum	Fasting
Stearic acid (SA)	(Ouchi et al., 2002)	No difference	Any AMD	11	10	Case-control	Plasma, erythrocytes	
	(Kabasawa et al., 2011)	No difference/ No difference	Dry AMD/ Advanced: Neovascular	31/ 166	143	Case-control	Serum	Fasting

Supplementary table 5: Factors involved in extracellular matrix remodeling

	Reference	Up/down/ no difference	Type of AMD	Cases (n)	Controls (n)	Study design	Matrix	Comment
MMP1	(Zeng et al., 2013)	No difference/ No difference	Early AMD/ Advanced: Neovascular	75/ 89	80	Case-control	Serum	
	(Guymer et al., 2015)	No difference/ No difference/ No difference	Early AMD/ Advanced: GA/ Advanced: Neovascular	18/ 21/ 23	19	Cross-sectional Case-control	Serum	
MMP2	(Chau et al., 2007)	No difference/ No difference	Early AMD/ Advanced: Neovascular	15/ 18	17	Case-control	Plasma	
	(Zeng et al., 2013)	No difference/ No difference	Early AMD/ Advanced: Neovascular	75/ 89	80	Case-control	Serum	Associated with PCV
	(Guymer et al., 2015)	No difference/ No difference/ No difference	Early AMD/ Advanced: GA/ Advanced: Neovascular	18/ 21/ 23	19	Cross-sectional Case-control	Serum	
MMP9	(Chau et al., 2007)	Up/ Up	Early AMD/ Advanced: Neovascular	15/ 18	17	Case-control	Plasma	
	(Zeng et al., 2013)	No difference/ No difference	Early AMD/ Advanced: Neovascular	75/ 89	80	Case-control	Serum	Associated with PCV
	(Guymer et al., 2015)	No difference/ No difference/ No difference	Early AMD/ Advanced: GA/ Advanced: Neovascular	18/ 21/ 23	19	Cross-sectional Case-control	Serum	

Supplementary table 6: Dietary factors

	Reference	Up/down/ no difference	Type of AMD	Cases (n)	Controls (n)	Study design	Matrix	Comment
Albumin	(Blumenkranz et al., 1986)	No difference	Advanced: Neovascular	26	23	Case-control	Serum	
	(Eye-Disease-Case-Control-Study-Group, 1992)	No difference	Advanced: Neovascular	421	615	Case-control	Serum	
	(Klein et al., 2003a)	Down	Early AMD	366	1995	Population-Based Cohort	Serum	Cardiovascular Health Study
	(Klein et al., 2003c)	No difference/ No difference/ Down	Early AMD/ Advanced: GA/ Advanced: Neovascular	?	?	Population-Based Cohort	Blood	Beaver Dam Eye Study, total n=3674, case-control ratios not presented
	(Klein et al., 2005)	No difference	Any AMD	188	195	Nested Case-control	Serum	Beaver Dam Eye Study, trend to downregulation (P=0.08)
Vitamin A (retinol)	(Blumenkranz et al., 1986)	No difference	Advanced: Neovascular	26	23	Case-control	Serum	
	(Eye-Disease-Case-Control-Study-Group, 1992)	No difference	Advanced: Neovascular	421	615	Case-control	Serum	
	(Sanders et al., 1993)	No difference	Any AMD	65	65	Case-control	Plasma	
	(West et al., 1994)	No difference	Any AMD	129	377	Case-control	Plasma	
	(Delcourt et al., 1999b)	No difference	Advanced: Any	38	2119	Population-based	Plasma	POLA, same population as (Delcourt et al., 1999a)
	(Simonelli et al., 2002)	No difference/ No difference	Early AMD/ Advanced: Any	19/ 29	46	Case-control	Plasma	
	(Michikawa et al., 2009)	No difference/ No difference	Early AMD/ Advanced: Any	32/ 8	682	Case-control	Serum	
	(Zhou et al., 2011)	No difference/ Down	Early AMD/ Advanced: Neovascular	92/ 82	89	Case-control	Serum	
Vitamin B12 (cobalamin)	(Heuberger et al., 2002)	No difference/ No difference	Early AMD/ Advanced: Any	329/ 16	3182	Population-based Cross-sectional	Serum	NHANES III
	(Kamburoglu et al., 2006)	Down/ No difference	Advanced: Neovascular/ Dry AMD	30/ 30	30	Case-control	Plasma	Lower in nAMD vs. dry AMD
	(Rochtchina et al., 2007)	Down	Advanced: Any	53	2910	Population-Based Cross-sectional	Serum	Blue Mountains Eye Study
	(Gopinath et al., 2013)	Down	Any AMD	219	1171	Cohort	Serum	Blue Mountains Eye Study, subgroup

								analysis showed significant downregulation in early and advanced AMD
	(Obeid et al., 2013)	No difference/ No difference	Advanced: Neovascular/ Dry AMD	31/ 38	48	Case-control	Plasma	All cataract subjects
Vitamin B9 (folate)	(Heuberger et al., 2002)	No difference	Early AMD	329	3182	Population-based Cross-sectional	Serum	NHANES III, Analysis for advanced AMD were not performed for folate because one or more quintiles had 0 cases
	(Klein et al., 2005)	No difference	Any AMD	188	195	Nested Case-control	Serum	Beaver Dam Eye Study
	(Kamburoglu et al., 2006)	No difference/ No difference	Advanced: Neovascular/ Dry AMD	30/ 30	30	Case-control	Plasma	
	(Rochtchina et al., 2007)	No difference	Advanced: Any	53	2910	Population-Based Cross-sectional	Serum	Blue Mountains Eye Study
	(Gopinath et al., 2013)	No difference	Any AMD	219	1171	Cohort	Serum	Blue Mountains Eye Study, subgroup analysis also did not show significant differences
	(Obeid et al., 2013)	No difference/ No difference	Advanced: Neovascular/ Dry AMD	31/ 38	48	Case-control	Plasma	All cataract subjects
Vitamin C (ascorbic acid)	(Blumenkranz et al., 1986)	No difference	Advanced: Neovascular	26	23	Case-control	Serum	
	(Eye-Disease-Case-Control-Study-Group, 1992)	No difference	Advanced: Neovascular	421	615	Case-control	Serum	
	(Eye-Disease-Case-Control-Study-Group, 1993)	No difference	Advanced: Neovascular	390	577	Case-control	Serum	
	(West et al., 1994)	No difference	Any AMD	129	377	Case-control	Plasma	
	(Delcourt et al., 1999b)	No difference	Advanced: Any	38	2119	Population-based	Plasma	POLA, same population as (Delcourt et al., 1999a)
	(Simonelli et al., 2002)	No difference/ No difference	Early AMD/ Advanced: Any	19/ 29	46	Case-control	Plasma	Lower in advanced vs early AMD
	(Yildirim et al., 2011)	No difference	Advanced: Neovascular	25	25	Case-control	Serum	
	(Shen et al., 2012)	Down/ No difference/ Down	Early/ Advanced: GA/ Advanced: Neovascular	21/ 13/ 22	34	Case-control	Serum	
Vitamin D	(Parekh et al., 2007)	Down/	Early AMD/	823/	6875	Population-based	Serum	NHANES III

		No difference	Advanced: Any	54		Cross-sectional		
	(Golan et al., 2011)	No difference	Any AMD	1045	8124	Cross-sectional	Serum	
	(Millen et al., 2011)	No difference	Early AMD	241	1046	Observational	Serum	CAREDS, included women only, a significant interaction with age was found
	(Morrison et al., 2011)	No difference	Advanced: Neovascular	50	50	Family-based	Serum	
	(Singh et al., 2013b)	No difference	Any AMD	129	49	Cross-sectional	Plasma	
	(Cho et al., 2014)	No difference/ No difference	Any AMD/ Advanced: Any	584/ 55	7315	Population-Based Cross-sectional	Serum	KNHANES
	(Itty et al., 2014)	No difference/ Down	Dry AMD/ Advanced: Neovascular	216/ 146	100	Case-control	Serum	Lower in nAMD vs. dry AMD
	(Kim et al., 2014b)	Up	Any AMD	1278	15767	Population-Based Cross-sectional	Blood	KNHANES, after correction for possible confounders there was no significant association
	(Park et al., 2014b)	No difference/ No difference	Early AMD/ Advanced: Any	959/ 88	12667	Population-based Cross-sectional	Serum	KNHANES
	(Cougnard-Gregoire et al., 2015)	No difference/ No difference	Early AMD/ Advanced: Any	269/ 63	365	Population-based	Plasma	ALIENOR
Vitamin E (a/y-tocopherol)	(Blumenkranz et al., 1986)	No difference	Advanced: Neovascular	26	23	Case-control	Serum	
	(Eye-Disease-Case-Control-Study-Group, 1992)	No difference	Advanced: Neovascular	421	615	Case-control	Serum	
	(Tsang et al., 1992)	No difference	Any AMD	80	86	Case-control	Serum	
	(Eye-Disease-Case-Control-Study-Group, 1993)	No difference	Advanced: Neovascular	391	578	Case-control	Serum	
	(Sanders et al., 1993)	No difference	Any AMD	65	65	Case-control	Plasma	Correlation cholesterol
	(West et al., 1994)	Down	Any AMD	129	377	Case-control	Plasma	
	(Mares-Perlman et al., 1995)	No difference/ Down	Any AMD/ Advanced: Neovascular	167/ 31	167	Case-control	Serum	Not significant after correction for serum cholesterol levels
	(Smith et al., 1997)	No difference/ No difference	Early AMD/ Advanced: Any	102/ 54	156	Case-control	Serum	Blue Mountains Eye Study, also no difference for pooled analysis of all AMD cases vs controls
	(Belda et al., 1999)	Down	Any AMD	25	15	Case-control	Serum	
	(Delcourt et al., 1999b)	No difference	Advanced: Any	38	2119	Population-based	Plasma	POLA, same population as (Delcourt et al., 1999a)
	(Simonelli et al., 2002)	No difference/	Early AMD/	19/	46	Case-control	Plasma	Lower in advanced vs early AMD

		Down	Advanced: Any	29				
	(Cardinault et al., 2005)	No difference	Any AMD	37	24	Case-control	Serum	Numbers reported in abstract differ from text and tables
	(Michikawa et al., 2009)	No difference/ No difference	Early AMD/ Advanced: Any	32/ 8	682	Case-control	Serum	Trend to downregulation of α-tocopherol in late AMD (P=0.056)
	(Shen et al., 2012)	No difference/ No difference/ No difference	Early/ Advanced: GA/ Advanced: Neovascular	21/ 13/ 22	34	Case-control	Serum	
Cadmium (Cd)	(Erie et al., 2007)	No difference	Any AMD	53	53	Prospective Case-control	Blood, urine	Positive association between urinary cadmium levels and AMD in smokers
	(Junemann et al., 2013)	Up	Dry AMD	12	11	Case-control	Aqueous	
	(Cho et al., 2014)	No difference/ Up	Any AMD/ Advanced: Any	584/ 55	7315	Population-Based Cross-sectional	Serum	KNHANES, only significant in univariate analyses, not in multivariate
	(Kim et al., 2014a)	Up	Any AMD	312	4621	Population-Based Cross-sectional	Blood	KNHANES
	(Wu et al., 2014)	Up	Any AMD	426	4964	Cross-sectional	Blood, urine	NHANES, urinary samples were available in 1548 participants only
	(Park et al., 2015)	No difference/ Up	Early AMD/ Advanced: Any	243/ 11	3611	Population-Based Cross-sectional	Blood	KNHANES
Lead (Pb)	(Cho et al., 2014)	Up/ Up	Any AMD/ Advanced: Any	584/ 55	7315	Population-Based Cross-sectional	Serum	KNHANES, only significant in univariate analyses, not in multivariate
	(Wu et al., 2014)	Up	Any AMD	426	4961	Cohort	Blood	NHANES, Not associated after correction
	(Hwang et al., 2015)	No difference	Any AMD	312	4621	Population-Based Cross-sectional	Blood	KNHANES, trend to upregulation (P=0.08)
	(Park et al., 2015)	Up/ Up	Early AMD/ Advanced: Any	243/ 11	3611	Population-Based Cross-sectional	Blood	KNHANES
Mercury (Hg)	(Cho et al., 2014)	No difference/ Up	Any AMD/ Advanced: Any	584/ 55	7315	Population-Based Cross-sectional	Serum	KNHANES, only significant in univariate analyses, not in multivariate
	(Park et al., 2015)	No difference/ Up	Early AMD/ Advanced: Any	243/ 11	3611	Population-Based Cross-sectional	Blood	KNHANES
Iron (Fe)	(Blumenkranz et al., 1986)	No difference	Advanced: Neovascular	26	23	Case-control	Serum	
	(Junemann et al.,	Up	Dry AMD	12	11	Case-control	Aqueous	

	2013)							
	(Wysokinski et al., 2013)	No difference	Any AMD	493	171	Case-control	Serum	
Copper (Cu)	(Cardinault et al., 2005)	Up	Any AMD	37	24	Case-control	Serum	Numbers reported in abstract differ from text and tables
	(Junemann et al., 2013)	Down	Dry AMD	12	11	Case-control	Aqueous	
Manganese (Mn)	(Junemann et al., 2013)	No difference	Dry AMD	12	11	Case-control	Aqueous	
	(Park et al., 2015)	No difference/ Down	Early AMD/ Advanced: Any	100/ 4	1521	Population-Based Cross-sectional	Blood	KNHANES
Zinc (Zn)	(Eye-Disease-Case-Control-Study-Group, 1992)	No difference	Advanced: Neovascular	421	615	Case-control	Serum	
	(Belda et al., 1999)	Down	Any AMD	25	15	Case-control	Serum	
	(Simonelli et al., 2002)	No difference/ No difference	Early AMD/ Advanced: Any	19/ 29	46	Case-control	Serum	
	(Junemann et al., 2013)	No difference	Dry AMD	12	11	Case-control	Aqueous	
	(Park et al., 2015)	No difference/ Down	Early AMD/ Advanced: Any	71/ 3	1033	Population-Based Cross-sectional	Blood	KNHANES
Selenium (Se)	(Eye-Disease-Case-Control-Study-Group, 1992)	No difference	Advanced: Neovascular	421	615	Case-control	Serum	
	(Tsang et al., 1992)	Down	Any AMD	80	86	Case-control	Serum	Significant in univariate analysis only, borderline significant in multivariate (P=0.07)
	(Eye-Disease-Case-Control-Study-Group, 1993)	No difference	Advanced: Neovascular	390	578	Case-control	Serum	
	(Mayer et al., 1998)	Down	Advanced: Neovascular	10	9	Case-control	Blood	
	(Junemann et al., 2013)	No difference	Dry AMD	12	11	Case-control	Aqueous	

Supplementary table 7: Hormones

	Reference	Up/down/ no difference	Type of AMD	Cases (n)	Controls (n)	Study design	Matrix	Comment
Leptin	(Everekliogl u et al., 2003a)	Down/ Down	Early AMD/ Advanced: Any	16/ 16	20	Case-control	Serum	Advanced AMD patients had significantly lower levels compared to early AMD
	(Seshasai et al., 2015)	Down	Any AMD	426	927	Population-Based Case-control	Serum	Singapore Indian Eye Study & Singapore Chinese Eye Study
	(Haas et al., 2015)	No difference	Advanced: Neovascular	54	46	Case-control	Blood	
Melatonin	(Rosen et al., 2009)	Down	Any AMD	43	12	Case-control	Urine	measured 6-sulfatoxymelatonin
	(Schmid-Kubista et al., 2009)	Up	Any AMD	50	19	Prospective Cross-sectional Observational	Serum	measured N-acetyl-5-methoxytryptamine
DHEAS	(Defay et al., 2004)	Up	Early AMD	?	?	Population-Based	Plasma	POLA, only women included in study, total n=708, case-control ratios not presented
	(Tamer et al., 2007)	Down/ Down	Dry AMD/ Advanced: Neovascular	75/ 67	64	Case-control Prospective	Serum	
	(Ulas et al., 2013)	No difference	Advanced: Neovascular	142	141	Cross-sectional	Serum	

Supplementary table 8: Factors related to comorbidities

	Reference	Up/down/ no difference	Type of AMD	Cases (n)	Controls (n)	Study design	Matrix	Comment
Cystatin C	(Klein et al., 2009)	Up/ Up/ No difference	Early AMD/ Advanced: Neovascular/ Advanced: GA	374/ 56/ 37	2286/ 3238/ 3243	Population-Based	Serum	Beaver Dam Eye Study
	(Chong et al., 2014)	No difference	Early AMD	221	5653	Population-Based Cross-sectional	Serum	Multi-Ethnic Study of Atherosclerosis, in normotensive participants the top 10% highest Cystatin C levels were associated with a higher risk of early AMD (P=0.049)
	(Aoki et al., 2015)	No difference/ No difference	Any AMD	185	295	Cross-sectional	Serum	Hatoyama Cohort Study
Creatinine	(Blumenkranz et al., 1986)	No difference	Advanced: Neovascular	26	23	Case-control	Serum	Fasting samples
	(Cackett et al., 2008)	No difference/ No difference	Early AMD/ Advanced: Any	169/ 21	3075	Population-Based Cross-sectional	Serum	Singapore Malay Eye Study
	(Boey et al., 2010)	No difference	Any AMD	177	2923	Population-Based Cross-sectional	Serum	Singapore Malay Eye Study
	(Javadzadeh et al., 2010)	No difference	Advanced: Neovascular	45	45	Case-control	Serum	
	(Javadzadeh et al., 2012)	No difference	Advanced: Neovascular	45	45	Case-control	Serum	Note: this is the same cohort as described by (Javadzadeh et al., 2010)
	(Obeid et al., 2013)	No difference/ No difference	Advanced: Neovascular/ Dry AMD	31/ 38	48	Case-control	Serum	All cataract subjects
	(Cho et al., 2014)	Down/ Up	Any AMD/ Advanced: Any	584/ 55	7315	Population-Based Cross-sectional	Serum	KNHANES, Only significant in univariate analyses, not in multivariate
	(Itty et al., 2014)	No difference/ No difference	Dry AMD/ Advanced: Neovascular	216/ 146	100	Case-control	Serum	
	(Park et al., 2014b)	Down/ No difference	Early AMD Advanced: Any	958/ 88	12665	Population-based Cross-sectional	Serum	KNHANES
BUN	(Blumenkranz et al., 1986)	No difference	Advanced: Neovascular	26	23	Case-control	Serum	
	(Klein et al., 2009)	No difference/	Early AMD/	392/	2420/	Population-Based	Serum	Beaver Dam Eye Study

		No difference/ No difference	Advanced: Neovascular/ Advanced: GA	62/ 39	3424/ 3432			
	(Cho et al., 2014)	Down/ No difference	Any AMD/ Advanced: Any	584/ 55	7315	Population-Based Cross-sectional	Serum	KNHANES , Only significant in univariate analyses, not in multivariate
	(Park et al., 2014b)	No difference/ No difference	Early AMD/ Advanced: any	958/ 88	12667	Population-based Cross-sectional	Serum	KNHANES
Glucose	(Blumenkranz et al., 1986)	No difference	Advanced: Neovascular	26	23	Case-control	Serum	Fasting samples
	(Eye-Disease-Case-Control-Study-Group, 1992)	No difference	Advanced: Neovascular	421	615	Case-control	Serum	
	(Smith et al., 1998)	No difference/ No difference	Early AMD/ Advanced: Any	240/ 72	3342	Population-Based Cross-sectional	Serum	Blue Mountains Eye Study, fasting
	(Delcourt et al., 2001)	No difference/ No difference	Early AMD/ Advanced: Any	730/ 38	1372	Population-Based	Plasma	POLA, Fasting samples
	(Cackett et al., 2008)	No difference/ No difference	Early AMD/ Advanced: Any	169/ 21	3075	Population-Based Cross-sectional	Serum	Singapore Malay Eye Study
	(Boey et al., 2010)	No difference	Any AMD	177	2923	Population-Based Cross-sectional	Blood	Singapore Malay Eye Study
	(Javadzadeh et al., 2010)	No difference	Advanced: Neovascular	45	45	Case-control	Serum	fasting
	(Javadzadeh et al., 2012)	No difference	Advanced: Neovascular	45	45	Case-control	Serum	Fasting Note: this is the same cohort as described by (Javadzadeh et al., 2010)
	(Jonas et al., 2012)	No difference	Early AMD	215	4319	Population-Based Cross-sectional	Serum	Central India Eyes and Medical Study, postprandial glucose, 8 late cases not analyzed
	(You et al., 2012)	No difference	Early AMD	?	?	Population-Based	Blood	Beijing Eye Study, total n = 3049, case-control ratios not presented
	(Kim et al., 2014a)	No difference	Any AMD	312	4621	Population-Based Cross-sectional	Blood	KNHANES
	(La et al., 2014)	No difference/ Down	Early AMD/ Advanced: Any	1034/ 95	13223	Population-Based Cross-sectional	Blood	KNHANES, Fasting, conflicting results in text and tables
	(Yang et al., 2014)	No difference	Early AMD	200	6377	Population-Based	Plasma	Handan Eye Study, fasting
	(Hwang et al., 2015)	No difference	Any AMD	312	4621	Population-Based Cross-sectional	Blood	KNHANES

HbA1c	(Boey et al., 2010)	No difference	Any AMD	177	2923	Population-Based Cross-sectional	Blood	Singapore Malay Eye Study
	(Cackett et al., 2008)	No difference/ No difference	Early AMD/ Advanced: Any	169/ 21	3075	Population-Based Cross-sectional	Serum	Singapore Malay Eye Study
	(Jonas et al., 2012)	No difference	Early AMD	215	4319	Population-Based Cross-sectional	Serum	Central India Eyes and Medical Study, postprandial glucose, only 8 late cases thus not analyzed
	(Ulas et al., 2013)	No difference	Advanced: Neovascular	142	141	Cross-sectional	Serum	Trend to downregulation in nAMD (P=0.062), fasting
	(Cho et al., 2014)	Down/ Down	Any AMD/ Advanced: Any	584/ 55	7315	Population-Based Cross-sectional	Serum	KNHANES , Only significant in univariate analyses, not in multivariate
	(Kim et al., 2014a)	Down	Any AMD	312	4621	Population-Based Cross-sectional	Blood	KNHANES
	(Kim et al., 2014b)	No difference/ Down	Early AMD/ Advanced: Any	1163/ 115	15767	Population-Based Cross-sectional	Blood	KNHANES
	(La et al., 2014)	Down/ Down	Early AMD/ Advanced: Any	1034/ 95	13223	Population-Based Cross-sectional	Blood	KNHANES, Fasting, conflicting results in text and tables
	(Zehetner et al., 2014)	No difference	Advanced: Neovascular	30	12	Case-control	Plasma	
	(Hwang et al., 2015)	Down	Any AMD	312	4621	Population-Based Cross-sectional	Blood	KNHANES
LDH	(Blumenkranz et al., 1986)	No difference	Advanced: Neovascular	26	23	Case-control	Serum	
	(Roh et al., 2008)	Up	Any AMD	235	9082	Case-control	Serum	Yonsei Eye Study, only significant in univariate analysis, not in multivariate
AST	(Blumenkranz et al., 1986)	No difference	Advanced: Neovascular	26	23	Case-control	Serum	
	(Cho et al., 2014)	Up/ No difference	Any AMD/ Advanced: Any	584/ 55	7315	Population-Based Cross-sectional	Serum	KNHANES, Only significant in univariate analysis, not in multivariate
ALT	(Blumenkranz et al., 1986)	No difference	Advanced: Neovascular	26	23	Case-control	Serum	
	(Cho et al., 2014)	No difference/ No difference	Any AMD/ Advanced: Any	584/ 55	7315	Population-Based Cross-sectional	Serum	KNHANES
Hepatitis B surface antigen (HBsAg)	(Roh et al., 2008)	Up	Any AMD	235	9082	Case-control	Serum	Yonsei Eye Study
	(Cho et al., 2014)	Up/	Any AMD/	584/	7315	Population-Based	Serum	KNHANES

		No difference	Advanced: Any	55		Cross-sectional			
	(Park et al., 2014b)	Up/ No difference	Early AMD Advanced: Any	52/ 5	446	Population-based Cross-sectional	Serum	KNHANES	
Amyloid beta (1-42)	(Obeid et al., 2013)	No difference/ No difference	Advanced: Neovascular/ Dry AMD	31/ 38	48	Case-control	Plasma	All cataract subjects	
	(Guymer et al., 2015)	Up/ Up/ Up	Early AMD/ Advanced: GA/ Advanced: Neovascular	18/ 21/ 23	19	Cross-sectional Case-control	Plasma	trend to increasing plasma levels as AMD stage advances	
	(Haas et al., 2015)	Up	Advanced: Neovascular	54	46	Case-control	Blood		
Amyloid beta (1-40)	(Guymer et al., 2015)	No difference/ No difference/ Up	Early AMD/ Advanced: GA/ Advanced: Neovascular	18/ 21/ 23	19	Cross-sectional Case-control	Plasma	Trend to upregulation in GA patients (P=0.064)	
	(Haas et al., 2015)	No difference	Advanced: Neovascular	54	46	Case-control	Blood		

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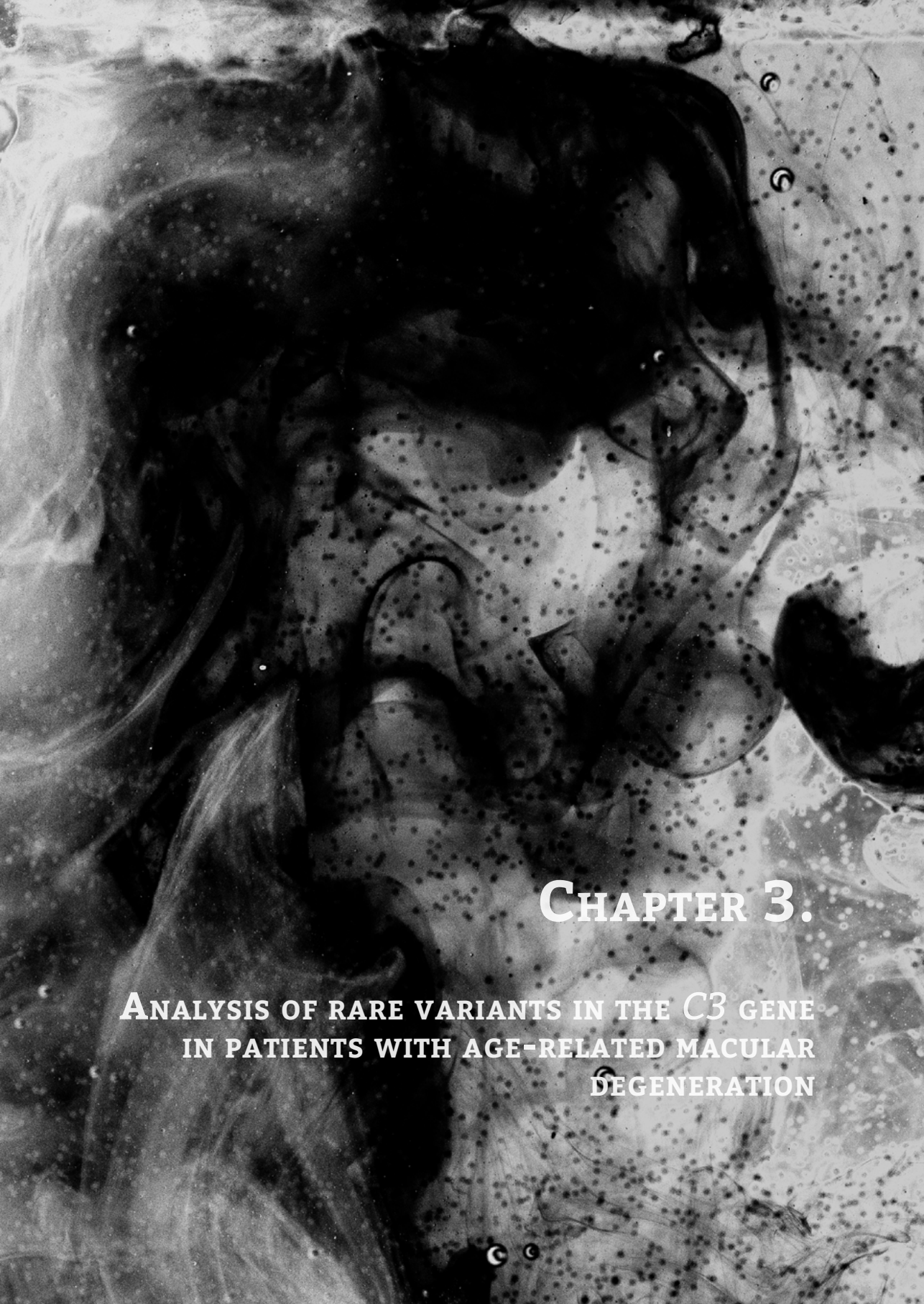

Chapter 3.

Analysis of rare variants in the *C3* gene in patients with age-related macular degeneration

Adapted from

Analysis of rare variants in the *C3* gene in patients with age-related macular degeneration

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ABSTRACT

Age-related macular degeneration (AMD) is a progressive retinal disorder affecting over 33 million people worldwide. Genome-wide association studies (GWASs) for AMD identified common variants at 19 loci accounting for 15–65% of the heritability and it has been hypothesized that the missing heritability may be attributed to rare variants with large effect sizes. Common variants in the complement component 3 (*C3*) gene have been associated with AMD and recently a rare *C3* variant (Lys155Gln) was identified which exerts a large effect on AMD susceptibility independent of the common variants. To explore whether additional rare variants in the *C3* gene are associated with AMD, we sequenced all coding exons in 84 unrelated AMD cases. Subsequently, we genotyped all identified variants in 1474 AMD cases and 2258 controls. Additionally, because of the known genetic overlap between AMD and atypical hemolytic uremic syndrome (aHUS), we genotyped two recurrent aHUS-associated *C3* mutations in the entire cohort. Overall, we identified three rare variants (Lys65Gln (P = 0.04), Arg735Trp (OR = 17.4, 95% CI = 2.2–136; P = 0.0003), and Ser1619Arg (OR = 5.2, 95% CI = 1.0–25; P = 0.05) at the C3 locus that are associated with AMD in our EUGENDA cohort. However, the Arg735Trp and Ser1619Arg variants were not found to be associated with AMD in the Rotterdam Study. The Lys65Gln variant was only identified in patients from Nijmegen, the Netherlands, and thus may represent a region-specific AMD risk variant.

3.1 INTRODUCTION

Age-related macular degeneration (AMD, MIM 603075) is a retinal disorder that causes progressive visual impairment in individuals aged over 50 years.[1] AMD primarily affects the macula, the central region of the retina, eventually leading to loss of central and sharp vision. It has been estimated that more than 33 million people suffer from vision loss due to AMD worldwide.[2] AMD is a multifactorial disease caused by a combination of genetic and environmental factors. GWASs identified common variants at 19 loci that influence disease susceptibility, accounting for 15–65% of the heritability.[3,4]

It has been hypothesized that the remaining genetic fraction influencing the risk for development of AMD, the so-called missing heritability, may be explained by rare, highly penetrant variants.[4] Simulation studies suggested that common variants are insufficient to account for disease burden in densely affected AMD families and that rare penetrant variants would offer a likely explanation.[5] In addition, a meta-analysis of AMD GWASs suggested that each of the 19 loci may harbour several independent variants associated to AMD susceptibility.[3] The rare variant hypothesis is supported by the identification of rare missense mutations in the fibulin-5 (*FBLN5*) gene and the hemicentin-1 (*HMCN1*) gene in AMD patients.[6,7] In addition, rare, highly penetrant variants in the genes encoding complement factor H (*CFH*), complement factor I (*CFI*), complement component 3 (*C3*) and complement component 9 (*C9*) have recently been found to be associated with AMD.[8-12]

Genetic studies have identified an important role for the complement cascade in the pathogenesis of AMD.[13] Interestingly, recent studies suggested a genetic overlap between AMD and atypical hemolytic uremic syndrome (aHUS), a life-threatening renal disease caused by chronic, uncontrolled activation of the complement system. It has been reported that 4–10% of aHUS patients carry mutations in the *C3* gene.[14] Moreover, disease-causing mutations previously identified in aHUS patients, such as Arg1210Cys in *CFH*, Gly119Arg in *CFI* and Lys155Gln in *C3* were found to confer a high risk of developing AMD.[8-12] However, the precise nature of this genetic overlap between two clinically distinct phenotypes remains unknown.

In this study, we explored the role of rare variants in the *C3* gene in the pathogenesis of AMD. We performed a two-stage analysis to identify rare variants in the *C3* gene. First, sequence analysis was carried out in a discovery set of 84 AMD cases

from the EUGENDA cohort, and subsequently the frequencies of these variants were determined in replication sets from EUGENDA and from the Rotterdam Study consisting of 1474 AMD cases and 2258 controls. In addition, two recurrent aHUS-associated *C3* mutations were genotyped in the entire AMD case-control cohort.[15,16]

3.2 RESULTS

To investigate the involvement of rare variants in the *C3* gene in AMD, the exons and flanking introns of *C3* were sequenced in a discovery cohort of 84 AMD cases (Table 1) from the Nijmegen area, the Netherlands. Sequencing identified three rare variants (MAF<1%; Lys155Gln/rs147859257, Arg735Trp/rs117793540 and Ser1619Arg/rs2230210) and two common variants (MAF≥1%; Arg102Gly/rs2230199 and Pro314Leu/rs1047286) (Table 2). None of the rare variants were found in 192 ethnicity-matched and age-matched controls. The Lys155Gln variant, which has recently been associated with AMD,[10-12] was found in five cases, while variants Arg735Trp and Ser1619Arg were found in one case each. Bioinformatic algorithms SIFT and PolyPhen predicted the variants Arg102Gly, Lys155Gln, and Pro314Leu not to be damaging whereas Arg735Trp and Ser1619Arg were predicted to be damaging to the protein function (Table 2).

Table 1. Demographics of studied subjects.

Variables	EUGENDA cohort		Rotterdam Study	
	Cases	Controls	Cases	Controls
Controls (n)		1246		1012
Intermediate AMD (n)	173		636	
Advanced AMD (n)	545		120	
Mean age (±SD)	76 ± 8	70 ± 5.9	80 ± 6.4	77 ± 6.5
Gender				
Male	271	532	354	497
Female	447	714	402	515

EUGENDA: a multicenter database comprising participants from Germany and the Netherlands

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Table 2. *C3* variants identified by sequence analysis of 84 AMD cases.

SNP ID	Sequence variants		Genotypes		Prediction algorithms	
	Nucleotide change	Amino acid change	Mm	mm	SIFT	PolyPhen2
rs2230199	c.304 C>G	Arg102Gly	34	11	Tolerated (0.5)	Tolerated (0)
rs1047286	c.941 C>T	Pro314Leu	30	10	Tolerated (0.1)	Tolerated (0.2)
rs147859257	c.463 A>C	Lys155Gln	5	0	Tolerated (0.2)	Benign (0.1)
rs117793540	c.2203 C>T	Arg735Trp	1	0	Deleterious (0)	Damaging (1)
rs2230210	c.4855 A>C	Ser1619Arg	1	0	Deleterious (0)	Damaging (0.8)

Major and minor allele indicated in capital and lower case respectively; Reference sequence of *C3* (NM_000064) gene; SIFT: Sorting Intolerant from Tolerant (Intolerance ≤ 0.05); PolyPhen2: Polymorphism Phenotyping (score 0→1)

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Next, the frequencies of the rare variants Arg735Trp and Ser1619Arg, as well as the common variants Arg102Gly and Pro314Leu were determined, in replication cohorts from EUGENDA and from the Rotterdam Study, consisting of 1474 AMD cases and 2258 controls of European ancestry (Table 3). In addition, two recurrent aHUS mutations (Lys65Gln and Arg161Trp) were included in the analysis (Table 3). The common variants Arg102Gly (OR = 1.2 [95% CI 1.1–1.4]; P = 0.001) and Pro314Leu (OR = 1.2 [95% CI 1.0–1.4]; P = 0.005) were significantly associated with AMD. In the EUGENDA cohort, the rare variant Arg735Trp was found heterozygously in 8 and homozygously in one out of 718 AMD cases and heterozygously in one out of 1246 controls (OR = 17.4 [95% CI 2.2–136]; P = 0.0003). However, in the Rotterdam Study the rare variant Arg735Trp was found heterozygously in 1 out of 785 AMD cases and heterozygously in 2 out of 1048 controls, and was thus not associated with the disease. Rare variant Ser1619Arg was found heterozygously in six out of 718 AMD cases and heterozygously in two out of 1244 controls in the EUGENDA cohort (OR = 5.2 [95% CI .1.0–25]; P = 0.05). In the Rotterdam Study the rare variant Ser1619Arg was found heterozygously in 6 out of 835 AMD cases and heterozygously in 11 out of 1279 controls, and was thus not associated with AMD. The aHUS mutation Lys65Gln was found heterozygously in three out of 717 AMD cases and was not observed in 1246 controls (P = 0.05) in the EUGENDA cohort, but was not identified in the Rotterdam Study. Arg161Trp was found heterozygously in two out of 644 AMD cases and was not observed in 1142 controls (P = 0.13) in the EUGENDA cohort. In the Rotterdam Study the aHUS mutation Arg161Trp was observed heterozygously in one out of 320 AMD cases and heterozygously in one out of 483 controls (P = 1.0) and was not significantly associated with AMD in the EUGENDA cohort nor in the Rotterdam Study.

Table 3. Genotyping of *C3* variants in EUGENDA and Rotterdam samples

SNP ID	Amino acid	EUGENDA cohort				Rotterdam Study				Combined cohorts (EUGENDA and Rotterdam)			
	change	MAF (%)		OR (95% CI)	p-value	MAF (%)		OR (95% CI)	p-value	MAF (%)		OR (95% CI)	p-value
		Controls	Cases		(2-sided)	Controls	Cases		(2-sided)	Controls	Cases		(2-sided)
Common variants													
Rs2230199	Arg102Gly	20.2	24.4	1.2 (1.1-1.4)	0.001	21.2	24	1.1 (1.0-1.3)	0.04	20.6	24.2	1.2 (1.1-1.3)	0.0002
Rs1047286	Pro314Leu	19.5	23.5	1.2 (1.0-1.4)	0.005	20.6	23.1	1.1 (0.9-1.3)	0.06	20	23.3	1.2 (1.0-1.3)	0.001
Rare variants													
	Lys65Gln	0	0.2	ND	0.04	0	0	NA	1	0	0.14	NA	0.05
	Arg161Trp	0	0.15	ND	0.13	0.1	0.15	1.5 (0.09-24)	1	0.03	0.15	5.0 (0.5-48)	0.14
rs117793540	Arg735Trp	0.04	0.69	17.4 (2.2-136)	0.0003	0.09	0.06	0.7 (0.06-8.4)	1	0.06	0.36	6.1 (1.7-21.6)	0.003
Rs2230210	Ser1619Arg	0.08	0.41	5.2 (1.0-25)	0.05	0.43	0.35	0.8 (0.30-2.2)	0.8	0.25	0.38	1.5 (0.6-3.2)	0.31

Major and minor allele indicated in capital and lower case respectively, MAF: Minor allele frequency, ND: OR could not be determined, NA: Not applicable, EUGENDA: a multicenter database comprising participants from Germany and the Netherlands

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To determine whether the identified variants conferred disease risk independent of the two known common *C3* variants (Arg102Gly/rs2230199 and Pro314Leu/rs1047286), a conditional logistic regression analysis was performed (Table 4). After conditioning on Arg102Gly/rs2230199, Arg735Trp remained associated with disease risk in the EUGENDA cohort (OR = 22.1, 95% CI = 2.8–173; P = 0.003). Similarly, after conditioning on Pro314Leu/rs1047286, Arg735Trp still showed association with disease risk in the EUGENDA cohort (OR = 22.0, 95% CI = 2.8–172; P = 0.003). In addition, Arg735Trp was significantly associated with disease risk in the EUGENDA cohort (OR = 22.1, 95% CI = 2.8–173; P = 0.003) after conditioning on both variants (Arg102Gly and Pro314Leu). Independent association with AMD could not be assessed for Lys65Gln and Ser1619Arg because too few data points were available to perform a reliable conditional analysis.

Table 4. Conditional analysis of Arg735Trp for SNPs Arg102Gly/rs2230199 and Pro314Leu/rs1047286

Condition	**EUGENDA cohort**		**Combined cohorts (EUGENDA and Rotterdam)**	
	OR (95%CI)	p-value	OR (95%CI)	p-value
Arg102Gly/rs2230199	22.1 (2.8-173)	0.003	6.2 (1.7-22.5)	0.005
Pro314Leu/rs1047286	22.0 (2.8-172)	0.003	6.2 (1.7-22.4)	0.005
Arg102Gly & Pro314Leu	22.1 (2.8-173)	0.003	6.2 (1.7-22.5)	0.005

EUGENDA: a multicenter database comprising participants from the Cologne area, Germany and the Nijmegen area, the Netherlands

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3.3 DISCUSSION

In this study, three rare variants (Lys65Gln, Arg735Trp and Ser1619Arg) in the *C3* gene were shown to be associated with AMD disease risk in our EUGENDA cohort. However, the Arg735Trp and Ser1619Arg variants were not found to be associated with AMD in the Rotterdam Study. The Arg735Trp and Ser1619Arg variants were also not associated with AMD in a recent study that analyzed the *C3* gene in 2,493 AMD cases and controls.[10] The Lys65Gln variant was only identified in AMD patients from the Nijmegen area, while it was not found in the Rotterdam Study, nor in a cohort from Boston.[10] The Lys65Gln variant may therefore represent a region-specific AMD risk variant, being confined to the east of the Netherlands, which is confirmed by the occurrence of Lys65Gln in Dutch aHUS patients.[10] A fourth variant, Arg161Trp, was found in three AMD cases and in one control; therefore it cannot be ruled out that the variant is in fact associated with AMD but a larger sample size would be required to detect a significant association.

The complement system plays an important role in the pathogenesis of AMD.[8-12] The *C3* gene encodes the complement component 3 protein, a central component of the complement cascade that plays a crucial role in clearance of pathogens and immune complexes. All three branches of the complement system, the classical pathway, the mannose-binding lectin pathway and the alternative pathway, converge at C3. At this stage, activation of C3 results in an amplification loop that ultimately forms a cytolytic membrane attack complex which targets pathogens. The system is tightly controlled by molecules like CFH and CFI. If the complement cascade is improperly regulated, host cells may also be subjected to complement attack, resulting in tissue damage and a spectrum of complement-mediated diseases.[17]

The rare variants reported in this study have previously been described for their association with aHUS, a chronic renal disorder caused by uncontrolled activation of the complement system, often leading to renal failure. Functional experiments have been performed in this context to determine the effect of the amino acid substitutions on C3 function. The Lys65Gln variant resides in the macroglobulin (MG1) domain of C3 and was shown to cause decreased binding to CFH.[16] Although not significantly associated in our study, the Arg161Trp variant was shown to cause hyperactive C3 convertase due to increased binding to factor B (CFB), and reduced binding to CFH and membrane cofactor protein (MCP; CD46), resulting in higher complement activity.[15,16,18]

Recombinant protein studies have shown that the Arg735Trp variant performs normal in binding and cleavage assays.[14] However, since the Arg735Trp residue is located in the anaphylatoxin (C3a) domain of C3, it is not likely to show an effect in the performed assays that evaluated binding and activation of the C3b fragment. Recent studies in animal models suggest that C3a anaphylatoxin has specific functions in the retina and retinal pigment epithelium, but it remains unknown how an amino acid substitution in C3a may contribute to the development of AMD.[19,20]

The Lys65Gln variant associated with AMD in this study has previously been associated with aHUS, further supporting the genetic overlap between AMD and aHUS. To rule out any renal pathology, the medical histories of AMD patients who carried this variant were evaluated, but no signs of aHUS or other renal pathologies were reported in these patients. Thus, although aHUS and AMD may overlap genetically, in this study no clinical overlap was shown, suggesting that compounding (genetic or environmental) factors contribute to a particular clinical phenotype. Such a notion is further supported by a study that showed that some aHUS patients carry multiple mutations in the complement factor genes.[21] In order to understand the shared associations, cross-phenotype studies are warranted to unravel the mechanisms common and unique to aHUS and AMD. This will lead to more rational approaches to diagnosis and therapy by targeting these specific molecular targets.

The identification of rare penetrant AMD-associated variants may have relevance for diagnostic, predictive and therapeutic purposes, although the exact interpretation may remain a challenge. Recent studies have identified several highly penetrant rare variants (Lys155Gln in *C3*, Gly119Arg in *CFI*, Pro167Ser in *C9* and Arg1210Cys in CFH), to be associated with AMD.[8-12] However, not all associations hold true among different populations. An example of this is the Arg1210Cys variant in *CFH* which was strongly associated in a North American AMD cohort8 but not in an Icelandic AMD cohort11 (Table 5). Genetic drift, founder effects or differences in genetic make-up that could compensate for a rare disruptive variant may underlie this phenomenon. In addition, the possibility that environmental effects could mask or enhance the penetrance of certain alleles between populations may also exist. To date, it remains unclear how these observations impact the predictive value of finding such variants in individuals. In contrast, variants that were proven to be functionally impaired and that are associated with AMD in several populations, such as the Lys155Gln variant in *C3*, have a much stronger predictive value.

Table 5. Rare variants in AMD studies

Gene	Variant	Study (Population)	Functional implication	Conclusions
Present study				
C3	Lys65Gln	NL	CFH binding ↓ [16]	Associated with AMD, not present in other populations, proven functionality, reported in aHUS [16]
	Arg161Trp	NL	CFH binding ↓, CFB binding ↑ [15, 16, 18]	Not associated with AMD, proven functionality, reported in aHUS [15, 16, 18]
	Arg735Trp	NL	Normal binding [14]	Not associated with AMD, normal functionality, reported in aHUS [14]
	Ser1619Arg	NL	NA	Not associated with AMD, not proven functionality, not found in aHUS
Other studies				
C3	Lys155Gln	ISL, NL, GER, USA [10, 11, 12]	CFH binding ↓ [18]	Associated with AMD, replicated in several populations, proven functionality, reported in aHUS [18]
CFI	Gly119Arg	NL, USA [9]	CFI activity ↓ [9]	Associated with AMD, replicated in different populations, proven functionality, reported in aHUS [9, 30]
C9	Pro167Ser	FRA, USA [10]	NA	Associated with AMD, replicated in different populations [10]
CFH	Arg1210Cys	USA [8]	C3b, heparin and endothelial cells binding ↓ [31]	Associated with AMD, not present in other populations, proven functionality, reported in aHUS [31]

NL: The Netherlands, ISL: Iceland, GER: Germany, USA: United States of America, FRA: France, NA: Not applicable

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In conclusion, we report a rare variant (Lys65Gln) at the *C3* locus in patients with AMD, while an association with two other variants (Arg735Trp and Ser1619Arg) was not confirmed in other cohorts. This study further supports that rare variants contribute to the genetic variance of AMD, which may have implications for predictive testing and personalized medicine in AMD.[10]

3.4 MATERIALS AND METHODS

3.4.1 Cases and controls

Cases and controls included in this study were selected from the population-based Rotterdam Study, Rotterdam, The Netherlands and from the European Genetic Database (EUGENDA), a multicenter database comprising participants from the Cologne area, Germany and the Nijmegen area, the Netherlands. All participants of this study underwent extensive retinal imaging which has been described in detail elsewhere[22,23]. In short, AMD staging was performed by grading of stereo fundus photographs according to the standard protocol of the Rotterdam grading center and Cologne Image Reading Center (CIRCL). All subjects were classified on the basis of the eye with the more severe diagnosis. Cases were aged ≥50 years of age and AMD was classified by the presence of at least 15 intermediate (63–124 μm) or at least one large drusen (≥125 μm) or geographic atrophy or choroidal neovascularization secondary to AMD. Control subjects were aged ≥65 years of age and did not have AMD (none or only small, hard drusen or only pigmentary abnormalities or less than 10 small drusen and pigmentary abnormalities). Written informed consent was obtained from all participants. The EUGENDA study was approved by the local research ethics committee, Commissie Mensgebonden Onderzoek Regio Arnhem-Nijmegen, the Netherlands, and Ethics Committee of the University Hospital Cologne, Germany. The Rotterdam study was approved by the institutional review board (Medical Ethics Committee) of the Erasmus Medical Center and by the review board of The Netherlands Ministry of Health, Welfare and Sports. The study was performed in accordance with the tenets of the Declaration of Helsinki.

3.4.2 Sequencing

Sanger sequencing of the *C3* (NM_000064) gene was performed in the discovery set, consisting of 84 AMD patients selected from the EUGENDA database. Primers were designed for all 41 coding exons and intron-exon boundaries by Primer3 software

(Supplementary Material, Table S1). Polymerase chain reaction (PCR) was performed, and PCR amplicons were sequenced using an automated sequencer (BigDye Terminator, version 3, 3730 DNA analyzer; Applied Biosystems). Sequences were assembled and analyzed using ContigExpress (Vector NTI Advance, Version 11.0, Life Technologies). Each newly identified variant was confirmed by a second independent PCR and bidirectional Sanger sequencing. The predicted effects of identified missense variants were examined using Polymorphism Phenotyping (PolyPhen)[24] and Sorting Intolerant from Tolerant (SIFT).[25]

3.4.3 Genotyping in the EUGENDA cohort

Variants (Lys65Gln, Arg102Gly, Arg161Trp, Pro314Leu, and Arg735Trp) were genotyped in the EUGENDA cohort using competitive allele-specific PCR assays (KASPar SNP Genotyping System, KBiosciences). KASPar genotyping was performed according to the manufacturer's protocol in a volume of 4 µl containing 10 ng of genomic DNA, 2.5 µl of 2× reaction mix, and 0.069 µl of assay. Thermal cycling conditions included a pre-incubation step at 94°C for 15 min, 20 cycles of 94°C for 10 s, 57°C for 5 s, 72°C for 10 s, followed by 23 cycles of 94°C for 10 s, 57°C for 20 s, 72°C for 40 s. Plates were analyzed on a 7900 Fast Real-Time PCR system (Applied Biosystems). The Ser1619Arg variant was genotyped in the EUGENDA cohort by Amplification Refractory Mutation System (ARMS)[26] PCR (Supplementary Material, Table S2). PCR reactions were performed in a volume of 12.5 µl using 20 ng genomic DNA, 1× buffer, 2.5 mM MgCl2, 1 mM deoxyribonucleotide triphosphates, 0.2 µM of each primer, and 0.5 U Taq DNA polymerase (Invitrogen, Life technologies). Thermal cycle conditions included a pre-incubation step at 95°C for 5 min, 16 cycles of 95°C for 30 s, 69°C for 30 s, 72°C for 45 s, followed by 25 cycles of 95°C for 30 s, 67°C for 30 s, 72°C for 45 s. PCR amplicons were analyzed by agarose gel electrophoresis. Each newly identified variant was confirmed by a second independent PCR and bidirectional Sanger sequencing.

3.4.4 Exome sequencing, exome chip analysis and genotyping in the Rotterdam Study

The occurrence of the C3 variants Lys65Gln, Arg161Trp, Arg735Trp, and Ser1619Arg in the Rotterdam Study (RS) was retrieved from exome chip and exome sequencing data. For exome sequencing purposes, genomic DNA of RS participants was prepared from blood and fragmented into 200–400 bp fragments using Covaris Adaptive Focused Acoustics (AFA) shearing according to the manufacturer's instructions

(Covaris, Inc., Woburn, MA). Illumina TruSeq DNA Library preparation (Illumina, Inc., San Diego, CA) was performed on a Caliper Sciclone NGS workstation (Caliper Life Sciences, Hopkinton, MA), followed by exome capture using the Nimblegen SeqCap EZ V2 kit (Roche Nimblegen, Inc., Madison, WI). This capture targets 44 Mb of exonic regions covering 30,246 coding genes, 329,028 exons and 710 miRNAs. Paired-end 2 ×100 sequencing was performed on Illumina HiSeq2000 sequencer using Illumina TruSeq V3 chemistry. Downstream analyses included demultiplexing (CASAVA software, Illumina), alignment using the burrows-wheeler alignment tool, followed by data processing and filtering with Picard, SAMtools and the Genome Analysis Tool-Kit.[27,28] Finally, variant detection was performed using GATK's Unified Genotyper. For exome chip analysis, DNA samples of the Rotterdam study were included in the joint calling experiment of the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium.[29] In short, DNA from the study participants was processed on the HumanExome BeadChip v1.0 (Illumina, Inc, San Diego, CA) querying 247,870 variable sites using standard protocols at seven genotyping centers. Each center genotyped a common set of 96 HapMap samples to be utilized for quality control and determination of batch effects. Raw datafiles for all samples were transferred to a central location and assembled into a single joint calling (Illumina GenomeStudio v2011.1 software and GenTrain 2.0 clustering algorithm). Call rates >99% were used for both study samples as for HapMap controls to define genotype clusters. Finally, 8994 variants were excluded for further analyses. Common variants (Arg102Gly and Pro314Leu) were genotyped in the RS cohort using Taqman assays (Applied Biosystems, Foster city, California, USA).

3.4.5 Statistical analysis

A Fisher's exact test was performed to assess the association between each variant and AMD, and also to check for Hardy-Weinberg equilibrium. P-values were calculated two-sided, and values of <0.05 were considered as statistically significant. Logistic regression analysis was performed to check for genotype interactions, to estimate the odds ratios (OR) with 95% confidence intervals (CI) and to adjust for age. Conditional analysis was performed to identify secondary association signals at the *C3* locus by accounting for two known AMD SNPs. Either Arg102Gly/rs2230199 or Pro314Leu/rs1047286 was added independently and combined to the regression model as a covariate to test the effect of the variant of interest. Data were analyzed using SPSS software, version 18.0.

3.5 SUPPLEMENTARY DATA

Table S1. List of *C3* gene sequencing primers

Primers	Sequence (5'- 3')	Product (bp)
Exon 1F	TGCTCACTCCTCCCCATC	199
Exon 1R	AAATGTCTGCTTCCACCCC	
Exon 2F	GGCGTCTCACATCCGTG	333
Exon 2R	GAAGACAGAAGGGGAGGGG	
Exon 3F	AGATCCGGAAGCTGGACC	444
Exon 3R	TTGCCTCTCCTAAGCCTGTG	
Exon 4F	AGCGGGTACCTCTTCATCC	300
Exon 4R	CCTTCCGGTGTGTCTTTCTC	
Exon 5-6F	TAGACACTGTGCACAGAGAAT	516
Exon 5-6R	TTTCTCTGTAGGCTCCACTAT	
Exon 7F	AAGATCCGAGCCTACTATGAA	311
Exon 7R	GTCCCCCACCTGGTCTTCACC	
Exon 8-9F	GGAGATCCCATTCTCCAGG	455
Exon 8-9R	TTTCTCTTCTGACCTGGTCTCC	
Exon 10-11F	GGAGGTCTAATCCTGAGGGG	500
Exon 10-11R	GACCCCACTGTGCAAACAC	
Exon 12F	CAGGTCTCAGGGATTCGG	349
Exon 12R	GAAGGAGTCCCAGGGGTG	
Exon 13F	GAGGCCAAGATCCGCTACTAC	546
Exon 13R	GACAGTTGAGAGACAGAGAGGG	
Exon 14F	AACCTTTCTGTCTTTCCACTC	422
Exon 14R	CATTCCCATCTTCAGCTTCAA	
Exon 15-16F	CACAGGTGCATATGTGGGG	629
Exon 15-16R	TCCCCTCCTCCCTCTCTG	
Exon 17F	GGGGAAGTCCTCCCTGG	360
Exon 17R	TCCCTCCTCAGACAGGAGTC	
Exon 18-19F	TTTCACCATGTTAGCTAGGCT	662
Exon 18-19R	AATGAGATGACACTCAGACAC	
Exon 20-21F	CTAAGAGCTGAGACCCAGGAG	585
Exon 20-21R	GAAGACCAGGAGCCCTCTC	
Exon 22-23F	TGCTGACCATCTGTGTGTCTG	421
Exon 22-23R	AATGAGATGGAATTTGGCTCC	

Exon 24F	AACCCTTTTCACGCCACC	343
Exon 24R	GGATCTTAGGGGAGGGATGC	
Exon 25F	TGAGTCCTTCCCTTTTTAAGG	406
Exon 25R	TCCGTGCTTAAGGATGCTTAA	
Exon 26F	GGTTGACATGGCAGTCTCTG	290
Exon 26R	CTCTCGTGTTCATCCTGCG	
Exon 27F	GATGACTGCCATGTGTGGAC	241
Exon 27R	CTGTGCTCTGCATCGGG	
Exon 28F	AAGTGCTGCTCGAATGATCC	297
Exon 28R	CAGTATCTCCCGCCCTGAAC	
Exon 29F	CTCTTTCTGAGCTTTCTCTGA	386
Exon 29R	AACTGATTCTCAACTCCACTG	
Exon 30-31F	GATTCTAGCCACTTTCCCAGG	495
Exon 30-31R	AGAGGAGATGGTCCCTCTGG	
Exon 32-33F	GACCATCTCCTCTTGTCCCC	432
Exon 32-33R	ACTTGGAAAGTACTGAATATCATGG	
Exon 34-35F	TCCTTGTCCAGGAACAGACC	424
Exon 34-35R	CCAGCCAGATAGAGGTCAGG	
Exon 36F	CAAGACAATGCTGGACTCCC	244
Exon 36R	CCCCACAATTCATATATACCTGG	
Exon 37-38F	TCTTTGGAGGGAGGCCC	504
Exon 37-38R	TGACAACCACACCTACCACC	
Exon 39-40F	TGCCCCTCATGGTCAAC	428
Exon 39-40R	ACAATGGTGTGGGCGTG	
Exon 41F	CCACACCATTGTCACGCC	280
Exon 41R	GGCAAAGAACTCCAGACACG	

Table S2. Genotyping probes: Ser1619Arg variant, Amplification Refractory Mutation System (ARMS) primer list

Primers	Sequence (5'- 3')	Product (bp)
Wild type-Forward	CCTGACCTGCCATTCTTCCCTCCAGCCTTA	298
Mutant type-Forward	CCTGACCTGCCATTCTTCCCTCCAGCCTTC	
Reverse	GGTTTCAAGTAGGATGGAGCTGAGCTGCAGGTG	

Table S3. List of kaspar primers

Variants	Allele X Primer	Allele Y Primer	Common Primer	Allele X	Allele Y
Lys65Gln	GTCCACGACTTCCCAGGCA	GTCCACGACTTCCCAGGCC	GTCTTCTCACTGGACAGCACTAGTT	A	C
Arg102Gly/rs2230199	CACGGTCACGAACTTGTTGCC	CACGGTCACGAACTTGTTGCG	GCCAACAGGGAGTTCAAGTCAGAAA	C	G
Arg161Trp	CAATGTTGACCATGACCGTCCG	CAATGTTGACCATGACCGTCCA	TTCACCGTCAACCACAAGCTGCTA	C	T
Pro314Leu/rs1047286	CCACCAGGTCTTCTGCTCGGA	CACCAGGTCTTCTGCTCGGG	TACTGCTGGACGGGGTGCAGAA	A	G
Arg735Trp/rs117793540	GCAACTACATCACAGAGCTGC	CTGCAACTACATCACAGAGCTGT	TGGCCAGGCCCAGGTGGCT	C	T

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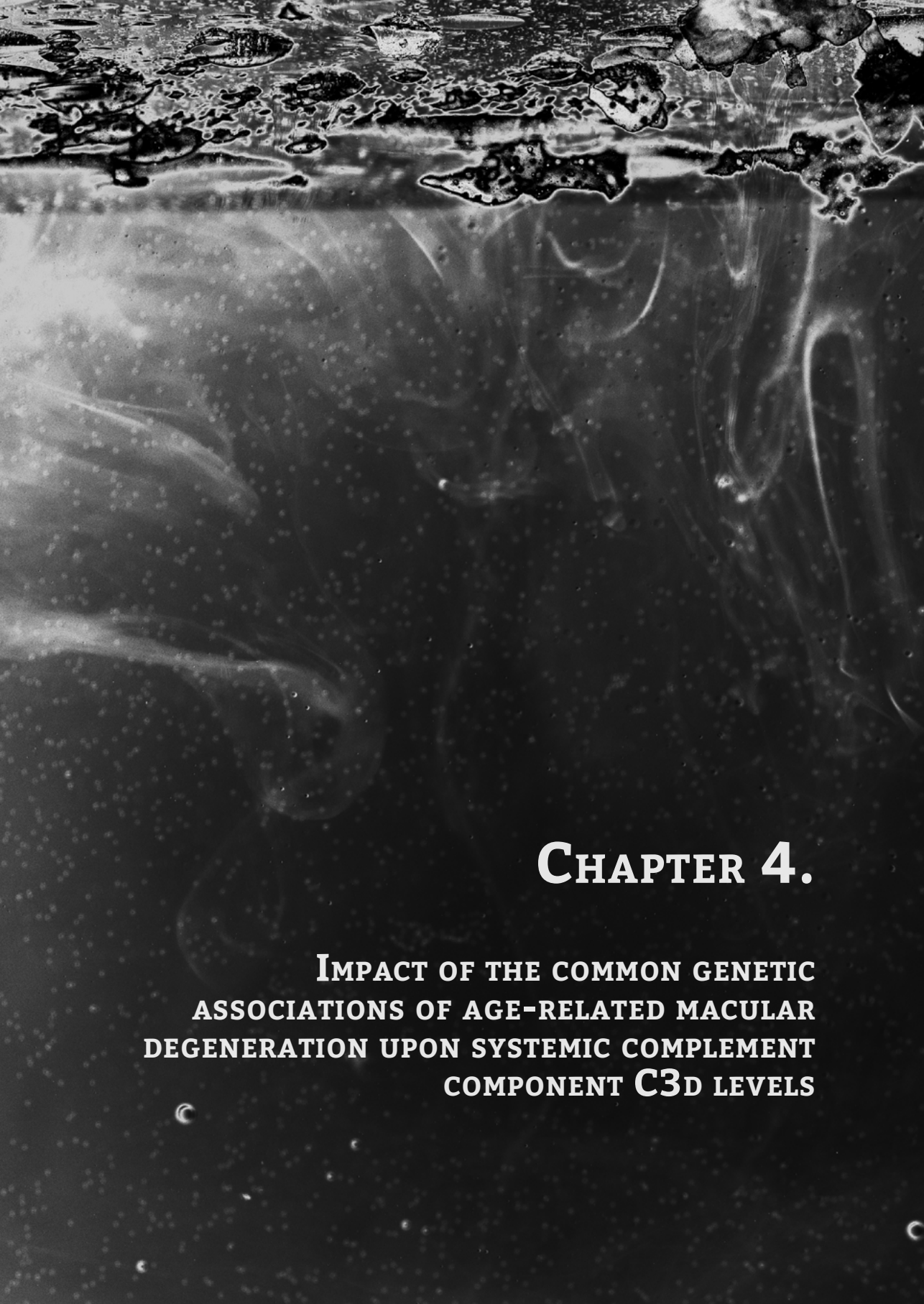

Chapter 4.

Impact of the common genetic associations of age-related macular degeneration upon systemic complement component C3d levels

Adapted from

Impact of the common genetic associations of age-related macular degeneration upon systemic complement component C3d levels

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ABSTRACT

Age-related macular degeneration (AMD) is a common condition that leads to severe vision loss and dysregulation of the complement system is thought to be associated with the disease. To investigate associations of polymorphisms in AMD susceptibility genes with systemic complement activation, 2655 individuals were genotyped for 32 single nucleotide polymorphisms (SNPs) in or near 23 AMD associated risk genes. Component 3 (C3) and its catabolic fragment C3d were measured in serum and AMD staging was performed using multimodal imaging. The C3d/C3 ratio was calculated and associations with environmental factors, SNPs and various haplotypes of complement factor H (*CFH*) genes and complement factor B (*CFB*) genes were analyzed. Linear models were built to measure the influence of genetic variants on the C3d/C3 ratio. The study cohort included 1387 patients with AMD and 1268 controls. Higher C3d/C3 ratios were found for current smoker ($p = 0.002$), higher age ($p = 1.56 \times 10^{-7}$), AMD phenotype ($p = 1.15 \times 10^{-11}$) and the two SNPs in the *C3* gene rs6795735 ($p = 0.04$) and rs2230199 ($p = 0.04$). Lower C3d/C3 ratios were found for diabetes ($p = 2.87 \times 10^{-6}$), higher body mass index ($p = 1.00 \times 10^{-13}$), the SNPs rs1410996 ($p = 0.0001$), rs800292 ($p = 0.003$), rs12144939 ($p = 4.60 \times 10^{-6}$) in *CFH*, rs4151667 ($p = 1.01 \times 10^{-5}$) in *CFB* and individual haplotypes in *CFH* and *CFB*. The linear model revealed a corrected R-square of 0.063 including age, smoking status, gender, and genetic polymorphisms explaining 6.3% of the C3d/C3 ratio. After adding the AMD status, the corrected R-square was 0.067. In conclusion, none of the evaluated genetic polymorphisms showed an association with increased systemic complement activation apart from two SNPs in the *C3* gene. Major genetic and non-genetic factors for AMD were not associated with systemic complement activation.

4.1 INTRODUCTION

Age-related macular degeneration (AMD) is a neurodegenerative disease causing visual impairment and blindness in the elderly population. Accumulation of drusen between Bruch´s membrane and the retinal pigment epithelium characterizes the early forms while the two advanced forms show geographic atrophy and choroidal neovascularization. Risk is multifactorial including environmental and genetic factors. Genetic variation accounts for up to 71% of the disease risk.[1] Many genetic polymorphisms were found in the alternative pathway of the complement system including complement factor H (*CFH*), complement component 3 (*C3*), Complement factor I (*CFI*), and complement factor B (*C2/CFB* locus) [2–8]. Complement proteins and their activation products have been identified in retinal deposits of AMD patients.[3,9-11]

The alternative complement pathway is constantly activated by the spontaneous hydrolysis of a thioester bond in C3 and a tight regulation including CFH is necessary to prevent excessive activation. It is hypothesized that a dysregulation of the complement system leads to tissue damage and finally AMD.

The dysregulation of the complement system or their activation fragments were also found systemically. In AMD patients, various components of the complement system were found at increased levels such as CFB, CFD, C3a, C5a, C3d, and Ba.[12,13]

While the association of genetic polymorphisms with AMD is well established, only polymorphisms in the *C3* gene and few haplotypes in the *CFH* and *CFB/C2* gene were found to be associated with complement activation products including factor C3d in two small cohorts.[12,13] The impact of other AMD susceptibility genes on the regulation of systemic complement activation remains unclear. In our study, we analyzed the association of 32 single nucleotide polymorphisms (SNPs) in or near 23 AMD risk genes with the C3d/C3 ratio as a marker for chronic complement activation in a Caucasian cohort of 2655 participants.

4.2 MATERIALS AND METHODS

4.2.1 Study population

2655 participants from the European Genetic Database (EUGENDA, www.eugenda.org) were included in the study. The study was performed in accordance with the tenets of the Declaration of Helsinki and the Medical Research Involving Human Subjects Act (WMO) and was approved by the local ethics committee of the University Hospitals in Cologne and Nijmegen. Written informed consent was obtained from all participants.

AMD staging was performed by grading of retinal images including stereo fundus photographs (FPs), fluorescein angiograms (FAs) and spectral domain optical coherence tomograms (SDOCTs) according to the standard protocol of the Cologne Image Reading Center (CIRCL) by certified graders (TR, LE). AMD was classified by the presence of pigmentary changes together with at least 10 small drusen (<63 µm) or the presence of intermediate (63–124 µm) or large drusen (≥125 µm diameter) in the Early Treatment Diabetic Maculopathy Study (ETDRS) grid or geographic atrophy and/or choroidal neovascularization (CNV) secondary to AMD in at least one eye.

Demographic data and non-genetic parameters including history of smoking (current/past/never), regular alcohol intake (yes/no), body mass index (BMI), arterial hypertension (yes/no), diabetes (yes/no), rheumatoid arthritis (yes/no), thyroid disease (yes/no), kidney disease (yes/no) and history of allergy (yes/no) were obtained by standardized interviewer-assisted questionnaires.

4.2.2 Complement component measurements and genetic analysis

Serum samples were used for C3d and C3 measurements. Serum was prepared by coagulation at room temperature. After centrifugation, the samples were stored at −80°C within 1 hour after collection. Complement component C3 and the activation fragment C3d were measured in serum samples as described previously.[14] The C3d/C3 ratio was calculated as a measure of C3 activation.

Genomic DNA was extracted from peripheral blood samples using standard procedures. Thirty-two SNPs in or near 23 AMD associated risk genes were chosen representing the majority of loci associated with AMD. Genotyping of SNPs in the

ARMS2 (rs10490924), *CFH* (rs1061170, rs800292, rs12144939, rs1410996), *CFI* (rs10033900, rs141853578), *C2* (rs9332739), *C3* (rs2230199, rs433594, rs6795735), *CFB* (rs4151667, rs641153), *CFD* (rs3826945), *LPL* (rs12678919), *LIPC* (rs10468017), *TIMP3* (rs9621532), *APOE2* (rs7412), *APOE4* (rs429358), *FADS1* (rs174547), *CETP* (rs2230199), *TLR* (rs4986790, rs3775291), *SERPING* (rs2511989), *ABCA4* (rs1800555, rs1800553, rs76157638), *VEGFA* (rs699946), *SPRYD7* (rs7995557), *COL8A1* (rs13081855), *COL10A1* (rs3812111), *SLC16A8* (rs8135665), *ADAMTS9-AS2* (rs6795735) genes were carried out as previously described.[15]

4.2.3 Haplotype analysis

In order to analyze the influence of haplotypes on C3d/C3 ratios, the posterior probability of each haplotype in the *CFH* gene including rs1061170, rs800292 and rs12144939 and in the *CFB* gene including rs4151667 and rs641153 was calculated using PHASE software, version 2.1.[16,17]

4.2.4 Statistical analysis

All calculations were performed using SPSS software version 21.0 (IBM Software and Systems, Armonk, NY, USA). C3d/C3 ratios are given as median and interquartile range (1st quartile – 3rd quartile). Due to the skewed nature of the data, the logarithm (log10) of the C3d/C3 ratios was used for analysis. Associations between logarithmic C3d/C3 ratios and genetic polymorphisms, haplotypes, phenotype and environmental factors were analyzed using t-tests or univariate analysis of variance (ANOVA) depending on number of variables. Associations between AMD phenotype and genetic polymorphisms were evaluated using logistic regression analysis. Linear models were performed to illustrate the influence of the genetic factors on complement activation. P-Values <0.05 were considered statistically significant.

4.3 RESULTS

4.3.1 Demographics and non-genetic factors

Mean age of the study population was 73.2±8.0 years (75.8±8.1 years for AMD patients and 70.4±6.8 years for controls, $p<0.001$). Demographic data, phenotype and environmental factors are summarized in Table 1. C3d/C3 ratios showed significant differences for age with increasing levels (except the youngest group from 50–59

Table 1. Median C3d/C3 ratios for non-genetic factors.

Non-genetic factor	N (%)	Median C3d/C3 ratio (IQR)	T-test/ univariate ANOVA
Female sex	1547 (58.3)	0.00424 (0.00325-0.00561)	0.90
Male sex	1108 (41.7)	0.00433 (0.00328-0.00567)	
Age 50-59 years	61 (2.3)	0.00430 (0.00337-0.00598)	$1.56x10^{-7}$
Age 60-69 years	879 (33.1)	0.00408 (0.00312-0.00550)	
Age 70-79 years	1140 (42.9)	0.00426 (0.00324-0.00547)	
Age 80-89 years	474 (17.9)	0.00462 (0.00356-0.00591)	
Age 90-99 years	97 (3.7)	0.00488 (0.00374-0.00705)	
No AMD	1268 (47.8)	0.00403 (0.00309-0.00536)	$1.15x10^{-11}$
AMD	1387 (52.2)	0.00449 (0.00348-0.00586)	
No arterial hypertension	1600 (63.6)	0.00428 (0.00328-0.00563)	0.24
Arterial hypertension	917 (36.4)	0.00425 (0.00321-0.00556)	
No diabetes	2268 (90.8)	0.00430 (0.00330-0.00567)	$2.87x10^{-6}$
Diabetes	231 (9.2)	0.00390 (0.00295-0.00495)	
No rheumatoid arthritis	2353 (93.5)	0.00426 (0.00327-0.00559)	0.35
Rheumatoid arthritis	164 (6.5)	0.00433 (0.00304-0.00564)	
No thyroid disease	2119 (84.2)	0.00426 (0.00326-0.00559)	0.77
Thyroid disease	398 (15.8)	0.00429 (0.00325-0.00559)	
No kidney disease	2403 (95.5)	0.00426 (0.00326-0.00559)	0.92
Kidney disease	114 (4.5)	0.00447 (0.00320-0.00572)	
No allergy	1984 (78.8)	0.00428 (0.00325-0.00562)	0.75
Allergy	533 (21.2)	0.00425 (0.00328-0.00552)	
Never smoker	1029 (43.0)	0.00431 (0.00325-0.00572)	0.002
Past smoker	1164 (48.6)	0.00415 (0.00320-0.00545)	
Current smoker	201 (8.4)	0.00451 (0.00355-0.00584)	
BMI <25	930 (40.1)	0.00464 (0.00360-0.00611)	$1.00x10^{-13}$
BMI 25-29	1084 (46.7)	0.00408 (0.00312-0.00531)	
BMI ≥30	308 (13.3)	0.00376 (0.00287-0.00493)	

IQR = interquartile range (1^{st} quartile – 3^{rd} quartile).

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years) and phenotype with higher values for AMD patients. A significant association was also found for diabetes, smoking, and BMI.

4.3.2 Associations of C3d/C3 levels with genetic polymorphisms

Significant associations of C3d/C3 levels were found in the *CFH* gene for the SNPs rs1410996, rs800292 and rs12144939, in the *CFB* gene for rs4151667 and in the *C3* gene for rs6795735 and rs2230199. In all SNPs of the *CFH* and *CFB* gene, these variants showed lower C3d/C3 ratios than the reference alleles, only variants in *C3* revealed higher values. After stratification in AMD cases and controls, associations for the major risk variants in rs1061170 (CFHY402H, $p = 0.35$ for no AMD; $p = 0.55$ for AMD) and rs10490924 (ARMS2, $p = 0.75$ for No AMD, $p = 0.25$ for AMD) with the C3d/C3 ratio could not be observed. A detailed analysis is outlined in Table 2.

Table 2. Median C3d/C3 ratios for single nucleotide polymorphisms (SNPs).

SNP	Homozygous non-variant (n)	Heterozygous variant (n)	Homozygous variant (n)	Median C3d/C3 ratio homozygous non-variant (IQR)	Median C3d/C3 ratio heterozygous variant (IQR)	Median C3d/C3 ratio homozygous variant (IQR)	Univariate ANOVA
ARMS2	GG=1039	GT=825	TT=241	0.00425	0.00416	0.00446	0.31
rs10490924				(0.00338–0.00589)	(0.00320–0.00556)	(0.00336–0.00593)	
CFH	TT=690	TC=967	CC=436	0.00425	0.00418	0.00433	0.66
rs1061170				(0.00320–0.00560)	(0.00323–0.00546)	(0.00335–0.00561)	
CFH	CC=459	CT=453	TT=113	0.00436	0.00404	0.00355	0.0001
rs1410996				(0.00334–0.00570)	(0.00310–0.00529)	(0.00209–0.00491)	
CFH	GG=1177	GA=608	AA=95	0.00427	0.00406	0.00394	0.003
rs800292				(0.00329–0.00567)	(0.00312–0.00535)	(0.00319–0.00516)	
CFH	GG=1290	GT=537	TT=64	0.00435	0.00390	0.00373	4.60×10^{-6}
rs12144939				(0.00329–0.00570)	(0.00305–0.00510)	(0.00287–0.00469)	
CFI	TT=499	TC=1056	CC=547	0.00426	0.00420	0.00424	0.80
rs10033900				(0.00324–0.00570)	(0.003.24–0.00553)	(0.00325–0.00547)	
CFI	GG=1862	GA=10	AA=0	0.00419	0.00485	N/A	0.18
rs141853578				(0.00321–0.00553)	(0.00415–0.00673)		
C2	CC=927	CG=70	GG=0	0.00422	0.00362	N/A	0.05
rs9332739				(0.00320–0.00544)	(0.00299–0.00492)		

C3	CC=1300	GC=713	GG=115	0.00415	0.00432	0.00432	0.04
rs2230199				(0.00319–0.00549)	(0.00334–0.00561)	(0.00327–0.00627)	
C3	CC=723	CT=900	TT=261	0.00420	0.00420	0.00421	0.81
rs433594				(0.00320–0.00557)	(0.00323–0.00555)	(0.00328–0.00548)	
C3	GG=1197	GA=617	AA=84	0.00413	0.00424	0.00448	0.04
rs6795735				(0.00317–0.00549)	(0.00334–0.00550)	(0.00333–0.00640)	
CFB	TT=1958	TA=160	AA=2*	0.00428	0.00358	0.00497	1.01×10^{-5}
rs4151667				(0.00327–0.00562)	(0.00297–0.00472)	(N/A)	
CFB	GG=1616	GA=265	AA=7*	0.00421	0.00414	0.00311	0.69
rs641153				(0.00324–0.00556)	(0.00314–0.00559)	(0.00223–0.00335)	
CFD	TT=488	TC=407	CC=93	0.00404	0.00421	0.00413	0.95
rs3826945				(0.00320–0.00542)	(0.00319–0.00531)	(0.00308–0.00525)	
LPL	AA=1705	AG=388	GG=31	0.00423	0.00423	0.00479	0.89
rs12678919				(0.00321–0.00561)	(0.00338–0.00542)	(0.00342–0.00593)	
LIPC	CC=1057	CT=888	TT=144	0.00421	0.00423	0.00441	0.46
rs10468017				(0.00322–0.00546)	(0.00322–0.00562)	(0.00337–0.00558)	
TIMP3	AA=882	AC=77	CC=7*	0.00411	0.00425	0.00392	0.70
rs9621532				(0.00318–0.00531)	(0.00332–0.00543)	(0.00376–0.00584)	
APOE2	CC=804	CT=160	TT=12	0.00420	0.00393	0.00417	0.53
rs7412				(0.00322–0.00543)	(0.00306–0.00524)	(0.00301–0.00485)	
APOE4	TT=689	TC=195	CC=9*	0.00420	0.00396	0.00272	0.49

rs429358				(0.00320–0.00537)	(0.00311–0.00530)	(0.00253–0.00609)	
FADS1	TT=995	TC=924	CC=209	0.00432	0.00416	0.00394	0.05
rs174547				(0.00330–0.00567)	(0.00319–0.00557)	(0.00334–0.00508)	
CETP	GG=997	GT=906	TT=246	0.00420	0.00424	0.00428	0.91
rs2230199				(0.00328–0.00559)	(0.00320–0.00546)	(0.00324–0.00568)	
TLR	AA=872	AG=119	GG=5	0.00419	0.00408	0.00283	0.24
rs4986790				(0.00319–0.00539)	(0.00333–0.00530)	(0.00248–0.00441)	
TLR3	CC=497	CT=380	TT=86	0.00401	0.00420	0.00430	0.31
rs3775291				(0.00306–0.00561)	(0.00333–0.00513)	(0.00320–0.00594)	
SERPING	GG=322	GA=485	AA=183	0.00410	0.00410	0.00428	0.56
rs2511989				(0.00317–0.00529)	(0.00322–0.00542)	(0.00316–0.00546)	
ABCA4	GG=985	GA=15	AA=0	0.00418	0.00368	N/A	0.17
rs1800555				(0.00319–0.00538)	(0.00337–0.00440)		
ABCA4	GG=975	GA=4	AA=0	0.00415	0.004.47	N/A	0.87
rs1800553				(0.00320–0.00538)	(0.00289–0.00659)		
ABCA4	GG=1854	CG=31	CC=0	0.00419	0.00443	N/A	0.81
rs76157638				(0.00321–0.00556)	(0.00332–0.00521)		
VEGFA	AA=629	AG=322	GG=42	0.00425	0.00400	0.00376	0.14
rs699946				(0.00327–0.00543)	(0.00303–0.00540)	(0.00324–0.00467)	
SPRYD7	TT=714	TC=220	CC=21	0.00418	0.00433	0.00390	0.30
rs7995557				(0.00322–0.00537)	(0.00317–0.00558)	(0.00255–0.00503)	

COL8A1	GG=1545	GT=333	TT=13	0.00421	0.00420	0.00502	0.73
rs13081855				(0.00322–0.00558)	(0.00321–0.00543)	(0.00342–0.00566)	
COL10A1	AA=742	AT=899	TT=241	0.00424	0.00411	0.00440	0.87
rs3812111				(0.00319–0.00555)	(0.00323–0.00548)	(0.00330–0.00575)	
SLC16A8	CC=1155	CT=632	TT=86	0.00421	0.00420	0.00423	0.83
rs8135665				(0.00324–0.00556)	(0.00319–0.00556)	(0.00312–0.00563)	
ADAMTS9	CC=647	CT=898	TT=343	0.00425	0.00419	0.00412	0.57
rs6795735				(0.00320–0.00572)	(0.00325–0.00540)	(0.00313–0.00561)	

*Due to small number of cases excluded from univariate ANOVA analysis; IQR = interquartile range (1st quartile – 3rd quartile).

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For the SNPs rs1061170, rs800292 and rs12144939 in the *CFH* gene and the SNPs rs4151667 and rs641153 in the *CFB* gene, haplotypes were associated with C3d/C3 levels (Table 3). All haplotypes were associated with lower C3d/C3 levels than the reference haplotype (Figure 1 and 2).

4.3.3 Associations of genetic polymorphisms with AMD

Performing logistic regression analysis, protective effects were found for variants in *CFH* rs1410996, *CFH* rs800292, *CFH* rs12144939, *CFB* rs641153 and *FADS1* rs174547. Variants in *CFB* rs4151667, *TIMP3* rs9621532 and *APOE4* rs429358 showed a trend for a protective effect on AMD without reaching statistical significance, which may be due to low minor allele frequencies or smaller effects of those SNPs.

Variants in *ARMS2* rs10490924, *CFH* rs1061170, *C3* rs2230199, *C3* rs6795735 and *CETP* rs2230199 were found to be associated with significantly higher risk for AMD (Table 4). Variants in *VEGFA* rs699946, *SLC16A8* rs8135665 and *ADAMTS9-AS2* rs6795735 also showed a trend for a higher AMD risk without statistical significance, which also may be due to a smaller effect on AMD development for each of those SNPs compared to *ARMS2* rs10490924 or *CFH* rs1061170.

Table 3. Haplotypes for *CFH/CFB* and median C3d/C3 ratios

Haplotype CFH	n	Haplotype frequency	MedianC3d/C3 ratio (IQR)	T-test
TGG	728	0.20	0.00456 (0.00345–0.00608)	-*
CGG	1544	0.42	0.00420 (0.00324–0.00550)	1.40×10^{-8}
TAG	755	0.20	0.00405 (0.00314–0.00530)	3.61×10^{-12}
TGT	620	0.17	0.00386 (0.00300–0.00504)	1.03×10^{-13}
CAG	21	0.008	0.00396 (0.00339–0.00507)	-**
TAT	18	0.005	0.00386 (0.00317–0.00513)	-**
Haplotype CFB	n	Haplotype frequency	MedianC3d/C3 ratio (IQR)	T-test
TG	3337	0.89	0.00424 (0.00325–0.00559)	-*
TA	279	0.07	0.00408 (0.00310–0.00546)	0.15
AG	152	0.04	0.00356 (0.00292–0.00471)	9.96×10^{-6}

For *CFH* single nucleotides polymorphisms rs1061170, rs800292, and rs12144939 and for *CFB* rs4151667 and rs641153 were chosen.

*T- test with comparison to reference haplotypes TGG and TG; **Due to small number of cases excluded from analysis; IQR = interquartile range (1st quartile – 3rd quartile).

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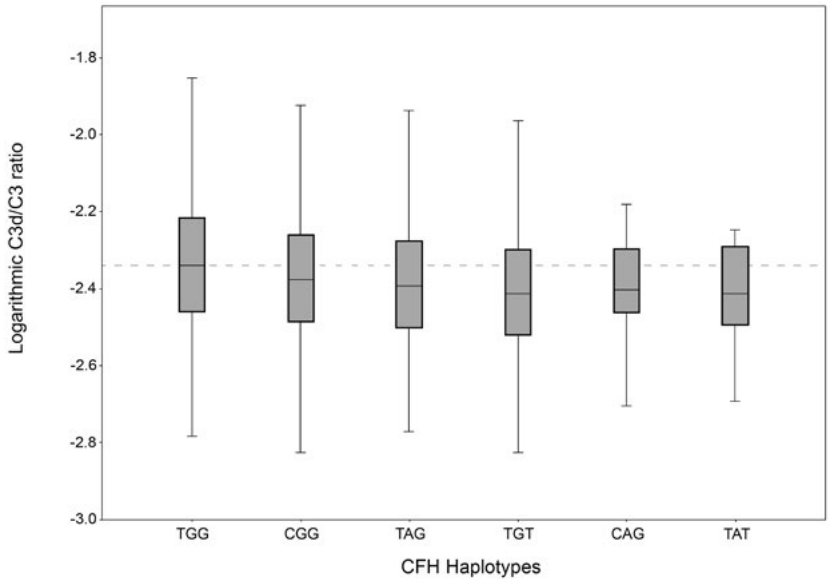

Figure 1. Logarithmic C3d/C3 ratios for haplotypes in the *CFH* gene rs1061170, rs800292 and rs12144939.
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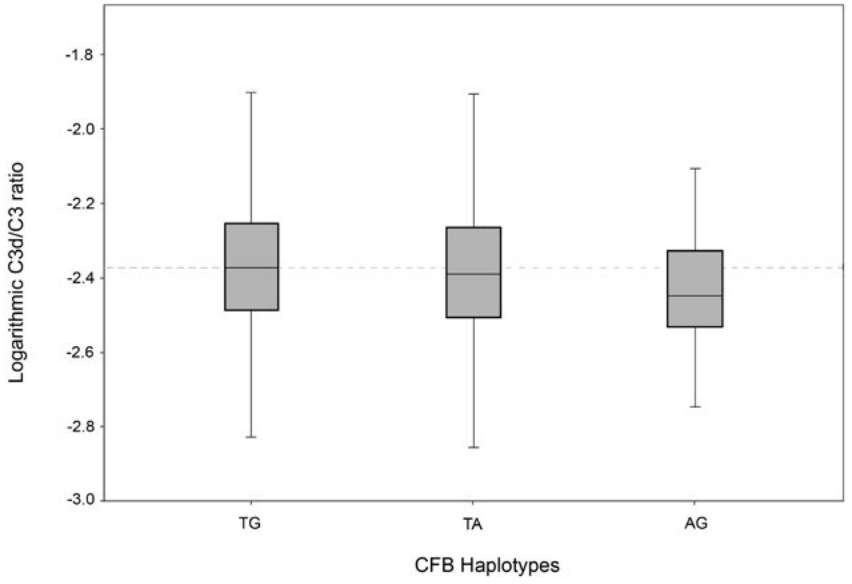

Figure 2. Logarithmic C3d/C3 ratios for haplotypes in the *CFB* gene rs4151667 and rs641153.
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Table 4. Logistic regression analysis between AMD and single nucleotide polymorphisms SNPs+.

SNP	Heterozygous variant			Homozygous variant		
	OR	95% CI	P-value	OR	95% CI	P-value
ARMS2 rs10490924	2.32	1.90–2.84	1.00×10^{-13}	8.13	5.66–11.69	1.00×10^{-13}
CFH rs1061170	1.57	1.27–1.95	3.16×10^{-5}	3.70	2.82–4.85	1.00×10^{-13}
CFH rs1410996	0.36	0.27–0.48	5.05×10^{-12}	0.23	0.15–0.36	1.28×10^{-13}
CFH rs800292	0.61	0.49–0.75	5.38×10^{-6}	0.70	0.44–1.10	0.12
CFH rs12144939	0.57	0.47–0.71	6.71×10^{-7}	0.51	0.28–0.91	0.02
CFI rs10033900	1.07	0.85–1.35	0.59	0.99	0.76–1.28	0.93
CFI rs141853578	1.17	0.96–1.43	0.12	-*	-*	-*
C2 rs9332739	0.70	0.42–1.17	0.17	-*	-*	-*
C3 rs2230199	1.17	0.96–1.42	0.11	2.17	1.42–3.31	0.0004
C3 rs433594	0.98	0.80–1.21	0.88	0.91	0.67–1.23	0.52
C3 rs6795735	1.10	0.89–1.36	0.37	2.06	1.27–3.33	0.03
CFB rs4151667	0.74	0.52–1.05	0.08	-*	-*	-*
CFB rs641153	0.72	0.54–0.96	0.02	-*	-*	-*
CFD rs3826945	1.01	0.76–1.34	0.94	0.76	0.48–1.22	0.26
LPL rs12678919	1.02	0.80–1.29	0.89	1.11	0.51–2.43	0.79
LIPC rs10468017	0.94	0.78–1.14	0.53	0.63	0.43–0.93	0.19
TIMP3 rs9621532	0.86	0.52–1.42	0.56	-*	-*	-*
APOE2 rs7412	1.21	0.84–1.76	0.31	-*	-*	-*
APOE4 rs429358	0.84	0.60–1.17	0.30	-*	-*	-*
FADS1 rs174547	0.88	0.72–1.06	0.18	0.64	0.46–0.88	0.006
CETP rs2230199	1.39	1.15–1.70	0.001	1.38	1.02–1.87	0.04
TLR rs4986790	1.08	0.71–1.64	0.71	0.53	0.08–3.36	0.50
TLR3 rs3775291	1.00	0.75–1.34	0.99	0.78	0.48–1.27	0.32
SERPING rs2511989	1.10	0.81–1.50	0.53	0.79	0.54–1.16	0.22
ABCA4 rs1800555	0.96	0.33–2.82	0.94	-*	-*	-*
ABCA4 rs1800553	0.88	0.12–6.58	0.90	-*	-*	-*
ABCA4 rs76157638	2.14	0.98–4.67	0.06	-*	-*	-*
VEGFA rs699946	1.08	0.81–1.44	0.61	1.47	0.75.2.89	0.27
SPRYD7 rs7995557	1.01	0.73–1.40	0.95	0.57	0.23–1.43	0.23
COL8A1 rs13081855	1.03	0.80–1.33	0.82	0.53	0.16–1.74	0.30
COL10A1 rs3812111	1.04	0.84–1.28	0.73	1.02	0.75–1.40	0.89
SLC16A8 rs8135665	1.21	0.98–1.49	0.08	1.39	0.87–2.21	0.17
ADAMTS9-AS2 rs6795735	1.21	0.98–1.51	0.08	1.30	0.98–1.72	0.07

+Adjusted for age and gender; *analysis not performed due to small group size.

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4.3.4 Linear Models

Linear models were composed based on univariate analysis of covariance (ANCOVA) with the logarithmic C3d/C3 ratio as dependent variable to evaluate the influence of various factors on the C3d/C3 ratio. In the first model, we included all SNPs (rs1410996, rs800292, rs12144939 in *CFH*, rs4151667 in *CFB*, rs6795735 and rs2230199 in C3) that had reached statistical significance in the individual analysis and the two major AMD risk SNPs risk *ARMS2* rs10490924 and *CFH* rs1061170. Additionally, age, gender, and smoking status was included. The corrected R-square was 0.063. Adding the AMD status to the model, the corrected R-square was 0.067.

In the second model, *CFH* haplotypes, age, gender, and smoking status were included. The corrected R-square was 0.038.

4.4 DISCUSSION

Dysregulation of the alternative complement pathway is thought to play a key role in AMD pathogenesis, which is also reflected by increased systemic complement levels.

In this study we analyzed the association of genetic AMD risk polymorphisms with systemic complement activation. We identified only a few variants in the *CFH, CFB*, and *C3* gene that showed an association with systemic complement activation, while for all other genetic polymorphisms associations were not observed.

While the association with genetic polymorphisms was weak, we found a significant association of the phenotype AMD with an increased C3d/C3 ratio which is in line with other smaller studies.[12,13] Our linear model including the AMD phenotype, the two major non-genetic risk factors age and smoking, and eight relevant SNPs could only explain 6.7% of the variation in the C3d/C3 ratio, indicating that these AMD risk polymorphisms do not explain sufficiently increased systemic complement activation found in AMD patients. The inclusion of *CFH* haplotypes in the model revealed an even lower explanation of the C3d/C3 ratio of only 3.8%.

Our analysis concentrated on C3d as a marker of chronic complement activation because it is a relatively stable protein with a long half-life. To correct for differences in the concentration of the precursor protein, we also measured C3 in the plasma.

CFH, CFB, and *C3* influence the regulation of the alternative pathway of the complement system. CFH acts as the major regulator of complement activation controlling the alternative pathway in blood and on cell surfaces[18], and accelerates the decay of the alternative C3 convertase (C3bBb).[19]

CFH is also a cofactor of CFI-mediated cleavage and inactivation of C3b.[20] The formation of C3d, a polypeptide fragment generated during alternative C3 convertase cleaves C3 to C3b, is also CFH dependent.[21] Alterations in the *CFH* gene may change the regulating characteristics of CFH resulting in an up or down regulation of the CFH dependent elements of the alternative complement pathway. In our cohort, SNPs rs1410996, rs800292, and rs12144939 in the *CFH* gene were associated with lower C3d/C3 ratios and a lower risk for AMD, whereas the most common AMD risk variant rs1061170 was not associated with the C3d/C3 ratio even after stratification in AMD patients and controls. Additionally, *CFH* haplotypes showed lower C3d/C3 ratios in all cases compared to the reference haplotype. Therefore, *CFH* SNPs were not associated with increased systemic complement activation.

CFB is an acute phase protein involved in the alternative complement pathway as a precursor of C3 convertase. CFB is cleaved to Bb which combines with C3b to form the alternative pathway C3 convertase C3bBb. An acute phase response-mediated up-regulation may result in elevated systemic plasma levels of CFB in AMD patients and may contribute to an enhanced systemic complement activity.[12,13,22] In our study we observed lower C3d/C3 ratios for the operatively protective *CFB* variants for AMD indicating that individuals with these polymorphisms show less complement activation.

Among all analyzed SNPs, only variants in the *C3* gene were associated with higher systemic C3d/C3 ratios which aligns with the results by Hecker et al.[11] The alternative pathway of the complement system starts with spontaneous hydrolysis of C3 and variants with a higher risk for AMD seem to influence this part of the complement cascade resulting in elevated systemic complement activation. Scholl et al did not observe a correlation between genetic variants in *C3* and systemic C3d levels[12] underlining that the systemic effects of AMD susceptibility genes on complement activation are only weak.

In our study, slightly higher C3d/C3 levels were found in AMD patients. In order to not miss a combined effect of multiple SNPs, we performed linear models to illustrate

the effect of the combination of SNPs on complement activation. These models could not explain the C3d/C3 ratio, showing that there have to be other systemic effects than AMD phenotype or genetic variants influencing systemic complement activation. Hecker et al also showed in a small cohort that risk haplotypes in *CFH* did not alter complement levels, whereas protective haplotypes reduced complement levels including C3d.[12]

A limitation of our study is the analysis of only two components of the complement system, which is accompanied by several strengths including a large cohort of well-balanced AMD patients and controls, a high number of investigated SNPs and the use of multimodal imaging that avoids misclassification of phenotypes.

In summary, we showed that the major AMD risk polymorphisms in *CFH* and *ARMS2* are not associated with increased systemic complement activation as measured by the C3d/C3 ratio. Few SNPs were associated with lower levels of systemic complement activation, particularly the *CFH* and *CFB* polymorphisms that are protective against AMD. Only variants in C3 were associated with elevated complement levels. Furthermore, a model including major genetic and non-genetic factors for AMD was not able to explain complement activation.

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Chapter 5.

A novel complotype combination associates with age-related macular degeneration and high complement activation levels in vivo

Adapted from

A novel complotype combination associates with age-related macular degeneration and high complement activation levels in vivo

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ABSTRACT

The complement system is the first line of defense against foreign intruders, and deregulation of this system has been described in multiple diseases. In age-related macular degeneration (AMD), patients have higher complement activation levels compared to controls. Recently, a combination of three single nucleotide polymorphisms (SNPs) in genes of the complement system, referred to as a complotype, has been described to increase complement activation in vitro. Here we describe a novel complotype composed of *CFB* (rs4151667)-*CFB* (rs641153)-*CFH* (rs800292), which is strongly associated with both AMD disease status ($p = 5.84 \times 10^{-13)}$ and complement activation levels in vivo ($p = 8.31 \times 10^{-9}$). The most frequent genotype combination of this complotype was associated with the highest complement activation levels in both patients and controls. These findings are relevant in the context of complement-lowering treatments for AMD that are currently under development. Patients with a genetic predisposition to higher complement activation levels will potentially benefit the most of such treatments.

5.1 INTRODUCTION

The complement system is part of our innate immunity where it acts as a first line of defense against foreign intruders[1] and as a surveillance system to discriminate between healthy host tissue, cellular debris and apoptotic cells[2]. The complement system can be triggered through one of its three pathways: the classical pathway (CP), the lectin pathway (LP) and the alternative pathway (AP). All three pathways converge at the level of complement component 3 (C3), which is cleaved into C3a (a potent proinflammatory molecule) and C3b (an opsonin).[1]

After C3 cleavage, a subsequent cascade of enzymatic reactions lead to the formation of the membrane-attack-complex, which is responsible for disrupting the target cell membrane by forming transmembrane pores[3]. Unlike the CP and the LP, which need certain triggers to become activated, the AP is always at a low level of activation via a process called "tick-over",[4] a spontaneous conversion of C3 to a bioactive form $C3(H_2O)$[5]. This conversion leads to a conformational change that allows for the binding of complement factor B (FB), similar to C3b[5] and, through a series of subsequent steps, forms the initial C3 convertase $C3(H_2O)Bb$[1]. This convertase cleaves C3 molecules into C3a and C3b[5,6]. In plasma, AP amplification is controlled by complement factor H (FH), which inactivates the C3 convertase and catalyses complement factor I (FI) degradation of C3b[7]. Dysregulation of the AP is associated with a number of diseases[8], a strong example being age-related macular degeneration (AMD).[9,10,11,12]

AMD is a progressive retinal disease that leads to vision loss in the elderly population[13]. It is a multifactorial disease caused by both genetic and environmental factors. Several lines of evidence support the involvement of the complement system in the pathology of AMD. Some of the highest genetic risk for AMD is conferred by single nucleotide polymorphisms (SNPs) in or near genes of the complement system.[14] Additionally, complement activation levels in plasma/serum are significantly higher in patients compared to controls[9,10,11,12] and complement components have been described in the composition of retinal deposits called drusen, which are a hallmark of the disease.[15]

Currently, AMD therapies that aim to inhibit or lower complement activation are being developed,[16,17] but it has been suggested that one of these inhibitors, lampalizumab, is effective only in a subset of patients that carry a specific genotype.[18] Therefore, it is important to understand the genetic risk factors that influence complement activation in order to identify those individuals that would benefit the most from such

treatments.

Several studies have evaluated the effect of SNPs on complement activity, and only moderate effects have been observed.[19,20,21] In vitro studies show that complement activity can increase six-fold when multiple SNPs in the complement system interact together.[20] Such combinations of SNPs in the complement system are called complotypes. Harris et al. defined the complotype as any inherited pattern of genetic variants in complement genes which alters risk for both inflammatory disorders and infectious diseases involving the complement system.[22] Until now, the best studied complotype in vitro is composed of three functional variants from the AP: *C3* (rs2230199 p.R102G), *CFB* (rs641153 p.R32Q) and *CFH* (rs800292 p.V62I). All three SNPs are individually associated with AMD.[23,24,25] Although the presence of all three SNPs led to markedly higher complement activity in vitro, the effect of the complotype has so far neither been investigated in human plasma samples, representative of the in vivo situation, nor been associated with any disease.

In a recent study, we have found another functional SNP in *CFB* (rs4151667) to be more strongly associated with complement activation[9] than the individual SNPs in the most studied complotype (*C3* (rs2230199), *CFB* (rs641153) and *CFH* (rs800292)). The aims of this study, therefore, are: 1) to expand the complotype with the *CFB* variant (rs4151667) we found to be highly associated with complement activity; 2) to evaluate the relation of the complotype with complement activation in human plasma samples, representative of the in vivo situation; and 3) to investigate the association between the complotype and AMD.

5.2 RESULTS

The study was performed in three consecutive steps. First, the individual associations of *CFH* (rs800292), *CFB* (rs4151667), *CFB* (rs641153) and *C3* (rs2230199) with AMD and with complement activation were verified. Next, we determined the most informative complotype for complement activation. Finally, we analyzed the association of the resulting complotype with the disease and with complement activation.

5.2.1 Individual association of *CFH* (rs800292), *CFB* (rs4151667), *CFB* (rs641153) and *C3* (rs2230199) with AMD and complement activation

In a previous study, *CFH* rs800292, *CFB* rs4151667, *CFB* rs641153 and *C3* rs2230199 were tested for their association with AMD in 2,655 individuals[9]. For the purpose of this study, 387 additional individuals were genotyped, amounting to a total of 3,042 subjects (1,615 AMD and 1,427 Controls). The mean age was 75 for AMD and 70 for controls. The gender distribution was: 41% males to 59% females. All four SNPs were significantly associated with AMD (Supplementary Table S1). SNPs *CFH* rs800292 (minor allele A), *CFB* rs4151667 (minor allele A) and *CFB* rs641153 (minor allele A) are protective, whereas the *C3* rs2230199 (minor allele C) infers increased risk of AMD.

To determine the association of these SNPs with complement activation levels, *CFH* (rs800292), *CFB* (rs4151667), *CFB* (rs641153) and *C3* (rs2230199) were included in a single general linear model, corrected for age, gender, body mass index (BMI) and disease status. The model revealed significant independent associations with complement activation levels for all four SNPs. Figure 1 illustrates P-values, mean log-transformed complement activation levels and genotype distribution for the four tested SNPs.

When we looked at the difference in mean complement activation level between the genotypes for each SNP, the high-risk *C3* (rs2230199) genotype (GG) showed higher complement activation levels than the heterozygous (CG) and ancestral (CC) genotype. The protective *CFH* (rs800292) genotype (AA) showed lower complement activation levels than the other genotypes (Figure 1). However, a statistically significant difference in mean complement levels was only observed between the heterozygous genotype (GA) and the major genotype (GG) ($p = 0.002$), presumably due to the limited number of individuals carrying the AA genotype. The protective *CFB* (rs641153) genotype (AA) and the heterozygous (GA) genotype displayed lower mean complement activation levels than the ancestral (GG) genotype. For *CFB* (rs4151667), the homozygous protective genotype for AMD (AA) could not be statistically compared to the homozygous ancestral genotype (TT), due to low number of individuals in this genotype group. The observed effects are driven by the difference in mean complement activation levels between the heterozygous (TA) genotype and the ancestral (TT) genotype (Figure 1).

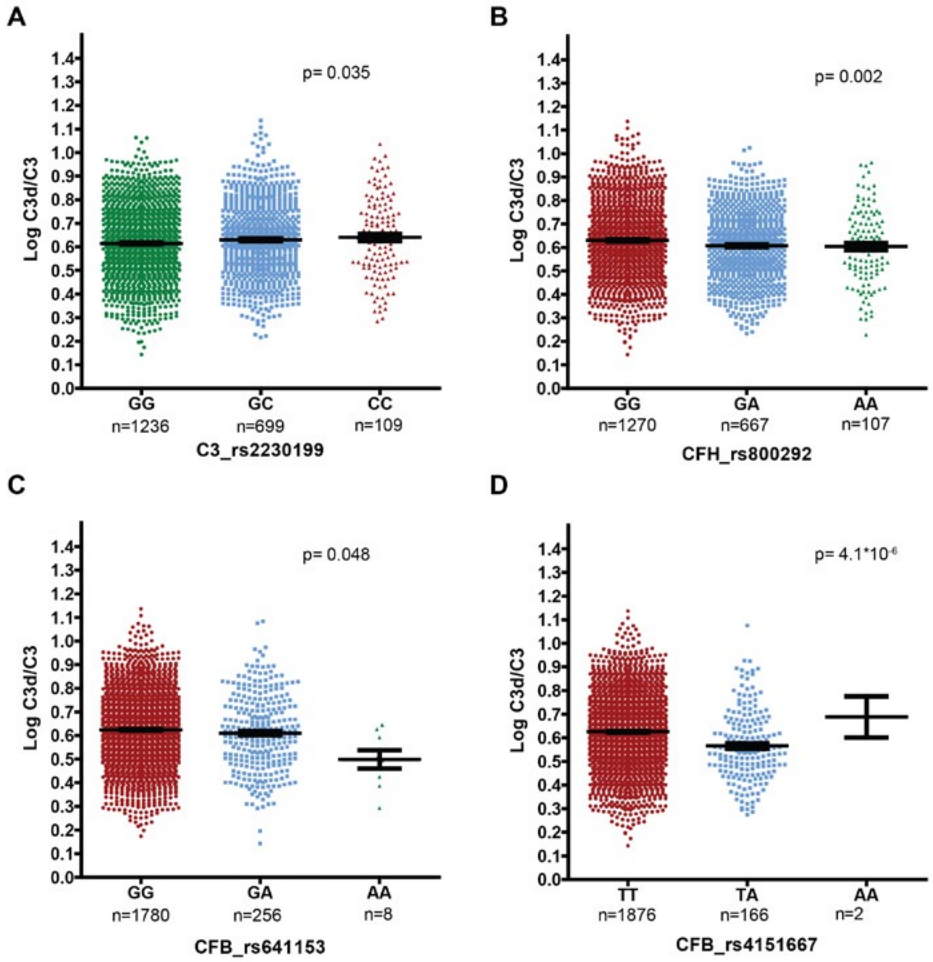

Figure 1. Plasma complement activation levels (log-transformed C3d/C3 ratio) for *C3*, *CFH* and *CFB* genotype groups. Each genotype per SNP is plotted on the X axis in an individual dot plot. The homozygous genotypes conferring increased risk for AMD are indicated in red; the homozygous genotypes that are protective for AMD are indicated in green. The number of individuals carrying a specific genotype is indicated below each genotype. The Y axis represents the Log-transformed C3d/C3 ratio level as a measure of complement activation. The p-values represent the overall significance for each SNP included in the model.

5.2.2 The most informative SNP combination in determining complement activation or AMD status

As all four SNPs were individually and independently associated with both complement activation and AMD status, the next step aimed to assess which combination of SNPs best predicted these associations. It was impossible to introduce genotype combinations of all 4 SNPs into the model because of the very low samples number of individuals in each of the resulting groups. For this reason, only combinations of 3 SNPs were considered.

In order to determine which combination of SNPs could best explain complement activation and disease status, two random forest analyses were performed. In the first analysis, the ratio of C3d/C3 as a measure of complement activation was used as the dependent variable, whereas the second analysis was classified on AMD disease status. Variable importance analyses in both tests revealed that the SNP combination composed of *CFB* (rs4151667)-*CFB* (rs641153)-*CFH* (rs800292) was the strongest predictor for both complement activation and AMD status (Table 1). For the purpose of clarity, this combination of SNPs will be referred to as the novel complotype in the remainder of the manuscript.

5.2.3 Association of the novel complotype with AMD

Mathematically, there are 27 possible genotype combinations for a complotype composed of three SNPs. To ensure a meaningful interpretation of the statistical analyses, we included only those genotype combinations that were represented by at least ten individuals in both the patients and controls group. In our cohort, we observed seven genotype combinations that met these criteria. The distribution of all genotype combinations in our cohort is shown in Supplementary Table S2.

To determine the association of the novel complotype with AMD, a logistic regression analysis was performed. A strong overall association of the novel complotype with AMD ($p = 5.84 \times 10^{-13}$) was observed. In our analysis of the genotype combinations within the novel complotype, the most frequent genotype combination found in controls (TT-GG-GG) was set as reference. The logistic regression analyses corrected for age and gender revealed that, in comparison to TT-GG-GG, the other six genotype combinations were protective for AMD (Table 2).

Table 1. Variable importance scores of *C3*, *CFB* and *CFH* genotypes and genotype combinations on complement activation levels and AMD status

Variables	%IncMSE	IncNode Purity	Mean Decrease Accuracy	Mean Decrease Gini
C3 (rs2230199)	5.33	0.06	6.59	1.85
CFB (rs4151667)	13.24	0.20	2.51	0.92
CFB (rs641153)	5.77	0.11	6.08	2.41
CFH (rs800292)	6.07	0.15	13.24	6.57
C3 (rs2230199) - *CFB* (rs4151667) - *CFB* (rs641153)	8.83	0.34	8.71	5.07
C3 (rs2230199) - *CFB* (rs4151667) - *CFH* (rs800292)	9.08	0.35	13.12	9.66
C3 (rs2230199) - *CFB* (rs641153) - *CFH* (rs800292)	10.88	0.33	13.84	11.85
CFB (rs4151667) - *CFB* (rs641153) - *CFH* (rs800292)	18.58	0.62	18.47	17.28
C3 (rs2230199) - *CFB* (rs4151667) - *CFB* (rs641153) - *CFH* (rs800292)	10.25	0.49	14.07	15.36

Mean decrease accuracy and mean decrease Gini measure variable importance in predicting disease status. %IncMSE and IncNode Purity are measures for variable importance in predicting complement activation. For all variables, the highest values represent the best predictors.

5

Table 2. Association between the novel complotype and AMD

Genotype combination for the novel complotype	N		P	OR	95% CI	
	Contols	AMD			Lower	Upper
TT - GG - GG	607	916	$5.84*10^{-13}$	-	-	-
TA - GG - GA	47	23	$1.01*10^{-5}$	0.3	0.174	0.51
TA - GG - GG	55	65	0.131	0.74	0.494	1.096
TT - GA - GA	74	47	$6.65*10^{-7}$	0.36	0.237	0.535
TT - GA - GG	112	106	$3.2*10^{-4}$	0.57	0.422	0.775
TT - GG - AA	59	48	0.007	0.56	0.373	0.856
TT - GG - GA	406	370	$7.32*10^{-9}$	0.58	0.48	0.696

The model was established by logistic regression analysis, corrected for age and gender. Bonferroni corrected threshold for statistical significance is $p<0.008$.

5.2.4 Association of the novel complotype with complement activation

Finally, to determine the association of the novel complotype with complement activation, a general linear model was built, corrected for age, gender, BMI and disease status. This model showed that the novel complotype was highly associated with complement activation levels ($p = 8.31 \times 10^{-9}$). When we compared the different genotype combinations with one another, the TT-GG-GG combination was associated with the highest mean complement activation levels (Figure 2). The difference in mean complement activation levels between all genotype combinations, tested in a post-hoc Bonferroni corrected manner, are presented in Supplementary Table S3. When comparing complement activation levels between AMD patients and controls, we only observed a significant difference for genotype combination TT-GG-GA (Figure 2).

5.3 DISCUSSION

In a large case-control study, we show that carrying multiple AMD protective genotypes for *CFB* (rs4151667), *CFB* (rs641153) and *CFH* (rs800292) leads to lower levels of complement activation in plasma compared to the most frequent genotype combination of these SNPs in control individuals. This novel complotype was identified as the most predictive SNP combination for determining both complement activation levels and AMD status. This combination of SNPs, drawn from an in vivo setting, is different from what has previously been suggested on the basis of in vitro data.[20]

It is well established that SNPs in complement components *C3*, *CFB* and *CFH* influence the risk for AMD.[24,25,26] In this study, we confirmed that four common functional SNPs, *CFH* (rs800292), *CFB* (rs4151667), *CFB* (rs641153) and *C3* (rs2230199) are associated with AMD. The minor alleles of the *CFH* and the *CFB* SNPs are protective,[23,24] whereas the minor allele of the *C3* SNP confers increased risk of AMD[25]. The well-known AMD SNP *CFH* (rs1061170; Tyr402His) was not included in this study because it was not associated with complement activation in our previous study[9]. This SNP was not described to alter AP convertase regulation, but rather it displays differential binding to C-reactive protein[27] and malondialdehyde[28].

Higher levels of systemic complement activation in patients compared to controls have been described in multiple studies.[9,10,12,29] As the proteins encoded by *CFH*, *CFB* and *C3*

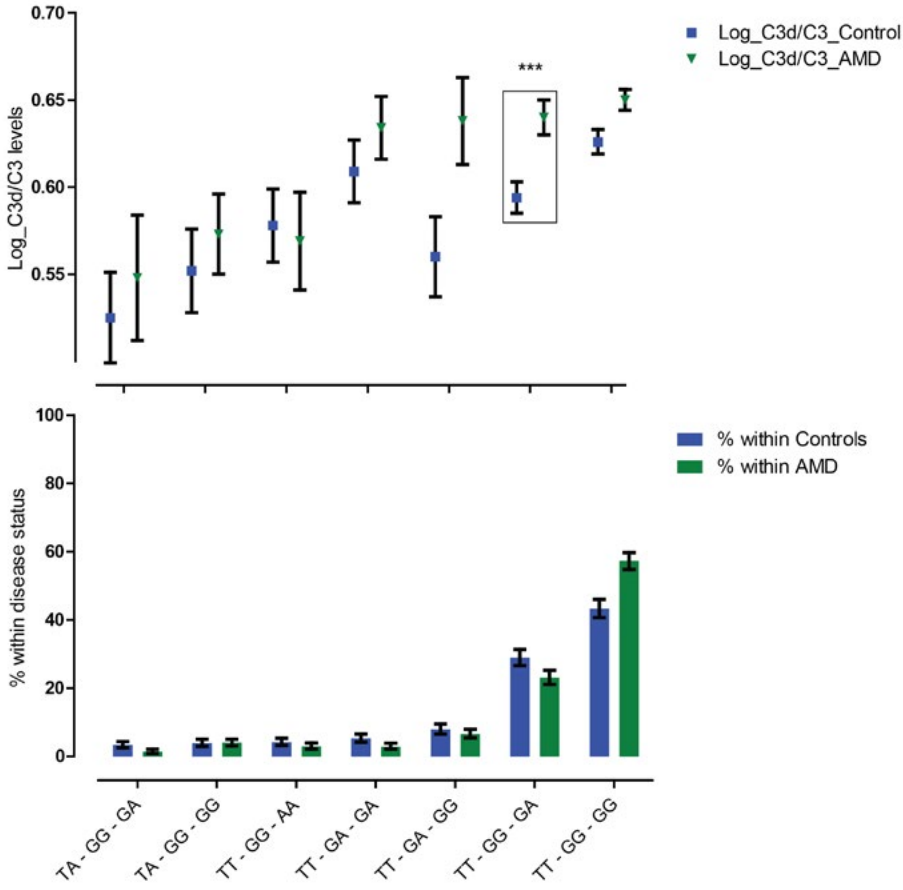

Figure 2. Mean C3d/C3 level and frequency of genotype combinations in AMD patients and controls. The blue and green bars represent the percentage of individuals carrying a specific genotype combination within their own disease status. The green triangles and blue squares represent mean C3d/C3 values for the corresponding genotype combination. The only genotype combination showing a significant difference in complement levels between AMD patients and controls was observed for TT-GG-GA with a p-value of $3*10^{-4}$ (after Bonferroni correction statistical significance is achieved at $p < 0.007$).

are key components of the AP of the complement system,[30] the contribution of these SNPs to disease susceptibility possibly comes from their impact on AP activation.

FH is a major regulator of the AP31. One of the ways in which it down-regulates complement activity is to bind C3b as a cofactor for its inactivation.[32] The A allele (p.62I) of the *CFH* (rs800292, p.V62I) SNP is a gain of function variant. In vitro experiments showed that the resulting protein binds more efficiently to C3b than the protein resulting from the G allele (p.V62) of this SNP,[19] thus leading to more complement inhibition. This is in line with our results, demonstrating that the *CFH* (rs800292) GG genotype was associated with decreased risk for AMD and lower levels of complement activation than the AA genotype.

FB binds hydrolyzed C3(H_2O) or C3b, which is then cleaved by complement factor D to form the AP C3 convertase that cleaves C3 to C3a and C3b,[22] thus fueling the AP amplification loop. The A (p.32Q) allele of rs641153 (p.R32Q) leads to a FB protein with decreased potential to form the C3 convertase and amplify complement activation[33]. The second *CFB* SNP (rs4151667) (p.L9H) resides in the signal peptide, and it has been proposed that it could alter CFB secretion.[24] In this study, the A alleles of both *CFB* SNPs were found to be protective for AMD and to lead to lower complement activation levels, even in heterozygous state, than the major homozygous genotype. The homozygous protective genotypes for *CFB* (rs4151667) were too rare for any reliable conclusions to be drawn.

C3 plays a central role in the complement system.[34] The G (p.102G) allele of *C3* (rs2230199, p.R102G) decreases the efficiency of regulation of C3b by FH, thus leading to an increase in complement activation. These observations are in accordance with the results in the present study, where the GG genotype is associated with risk for AMD and displays higher levels of complement activation than the CC genotype (Figure 1A). Even though it plays such an important role, it was not part of the most predictive complotype in the present study.

Several in vitro studies have shown that having multiple SNPs in complement genes would lead to higher complement activation.[20,35] In the present study, the novel complotype composed of *CFB*(rs4151667)-*CFB*(rs641153)-*CFH*(rs800292) had a larger effect on complement activation than the initially studied complotype *C3* (rs2230199)-*CFB* (rs641153)-*CFH* (rs800292)[20] (Table 1). The higher predictive value of the newly described complotype with respect to AMD might be related to

the fact that it is composed of protective SNPs only rather than of a combination of polymorphisms with opposing effects on AMD susceptibility. When comparing the strongest effect (OR = 0.3) of this new complotype on the risk of AMD with the odds ratios of the 38 individual loci described in the newest AMD GWAS[36], we notice that the effect size is close to both the *CFH* (OR = 0.38) and the *ARMS2* (OR = 2.81), albeit reverse, locus. It is worth mentioning that the OR of 0.3 for the complotype it is seen when comparing TA-GG-GA to TT-GG-GG which has only two alleles difference out of the six.

This study is the first to analyze this specific complotype combination for its association with AMD and complement activity. Although it would have been interesting to study the simultaneous presence of all four genotyped SNPs, cohorts even larger than ours are needed to avoid the problem of small genotype combination groups that cannot be reliably compared.

Intriguingly, the homozygous genotypes associated with the highest complement activation levels in all three SNPs (TT-GG-GG) in the novel complotype are found most frequently in both AMD patients and controls. This is in contrast to what was proposed in the theoretical model from[22], where the extreme genotype combinations were expected to be at the lower end of the carrier frequency spectrum. With fewer than ten individuals for patients or controls, the combination of all heterozygous genotypes was rare. The combination where all SNPs had the homozygous protective genotypes (AA-AA-AA) was not present in our cohort at all. In our study, therefore, the frequency distribution is skewed towards complement-raising genotypes.

This could potentially be explained by the fact that our cohort has a mean age of 73 years and might be enriched, therefore, for alleles that promote survival. In this case, the alleles that give higher complement activation could offer better lifetime protection against infection. However, these same genetic variants would potentially induce low-grade inflammation, and its effect would only be observed later in life, as is the case for AMD, a disease that is prevalent in the elderly population. In support of this hypothesis, immune genes have been described to have the highest rate of positive selection.[37] Upon examination of the amino acid conservation of the SNPs in the present study, in humans three complement-raising variants are the reference amino acid, compared to only one in primates (Supplementary Table S4).

A significant difference in complement levels was observed between AMD patients and controls carrying the TT-GG-GA genotype combination. Although the highest mean difference was observed between the groups carrying the TT-GA-GG combination, this difference was not significant due to the high standard error.

The four most prevalent genotype combinations are all associated with high levels of complement activation in AMD patients with only minor differences between the groups. The three genotype combinations that are least prevalent are associated with lower complement activity. If we look at the specific genotype combinations, some interesting observations can be made.

First of all, the TT-GG-GG genotype combination is associated with the highest complement activation levels and is more prevalent in AMD patients (57.3%) than in controls (43.3%). The TA-GG-GG genotype, which is only different with respect to 1 risk allele in *CFB* rs4151667, is at the lower end of complement activation. The only other genotype combination with TA instead of TT for *CFB* rs4151667 is also associated with lower complement activation. This suggests that this SNP might be the most important of the three SNPs in the novel complotype and is the driving force behind the influence on complement activation. This is also evident in the results from the random forest analyses, where *CFB* rs4151667 is the strongest predictor for complement activation compared to the other individual SNPs. Another interesting observation from Figure 1 is the difference in complement activation between genotype combinations TT-GG-AA and TT-GA-GA. Both combinations include four risk alleles and two protective alleles, but the difference in complement activation is striking, especially in the AMD group. Perhaps the presence of two protective alleles in one SNP, as in the TT-GG-AA genotype combination, has a stronger influence on complement activity than the combination of two heterozygous SNPs (TT-GA-GA). Observations like ours may help to clarify this and warrant further research, preferentially in an even larger dataset.

One of the major strengths of this study is the use of the large EUGENDA dataset. To the best of our knowledge, this is one of the largest datasets of complement activation to date. For the evaluation of mean differences in complement activation at a population level, as we have done in this study, a single measurement of C3 and C3d in each individual is sufficient. However, if complement activation would be used on an individual basis, such as for the selection of patients for clinical trials, multiple measurements over time would be preferred to correct for individual variations in complement activation.

In conclusion, the current study has demonstrated that a novel complotype composed of *CFB* (rs4151667) in combination with *CFB* (rs641153) and *CFH* (rs800292) is strongly associated with complement activation and AMD status. These findings are relevant in the context of future complement-lowering treatments for AMD. In the era of personalized medicine, we are moving towards a more individualized approach to the treatment of diseases. To evaluate new treatment strategies, we need detailed information to determine how subgroups of patients with a higher treatment response potential should be defined. In this case, genotype-based patient stratification may identify those individuals that are genetically predisposed to having the highest complement levels, potentially making them the best candidates for complement-inhibiting therapies in AMD.

5.4 MATERIALS AND METHODS

5.4.1 Study population

In this study, 3042 participants from the European Genetic Database (EUGENDA, www.eugenda.org), over the age of 50 years, were included. The study was performed in accordance with the tenets of the Declaration of Helsinki and the Medical Research Involving Human Subjects Act (WMO) and was approved by the local ethics committee of the University Hospitals in Cologne and Nijmegen. Written informed consent was obtained from all participants.

AMD and control status were assigned by multimodal image grading that included stereo fundus photographs, fluorescein angiograms and spectral domain optical coherence tomograms. The grading was performed according to the standard protocol of the Cologne Image Reading Center (CIRCL) by certified graders (TR, LE) as previously described[38].

Age, gender and BMI were obtained by standardized interviewer-assisted questionnaires.

5.4.2 Complement measurements and genetic analysis

Complement component C3 and the activation fragment C3d were measured in serum samples as previously described[9], and the C3d/C3 ratio was calculated as a measure of complement activation. The complement activation data were skewed and had

several outliers at the high end of the value range. In order to reduce the risk of outlier effects distorting the data, five percent of the highest values from the entire dataset were excluded from our analysis. After the exclusion of the outliers, the remaining skewedness of the C3d/C3 data was normalized by Log10 transformation.

Genomic DNA was extracted from peripheral blood samples using standard procedures. Four SNPs, *CFH* (rs800292), *CFB* (rs4151667), *CFB* (rs641153) and *C3* (rs2230199) were genotyped using the KASPar SNP Genotyping System by LGC Genomics.

5.4.3 Statistical analysis

All associations were calculated using SPSS software version 20.0 (IBM Software and Systems, Armonk, NY, USA). Associations with complement activation were analyzed using General Linear Models with C3d/C3 as the dependent variable. The models were corrected for age, gender, BMI and disease status.

The associations between AMD phenotype and the individual SNPs or the complotype were evaluated using logistic regression. To determine if the SNPs were independently associated with the disease, all four SNPs were included in the logistic regression model at once.

To avoid being relevant only to our sample set (overfitting), the most informative complotype combination was determined by calculating the variable importance in a random forest analysis using the R package (RandomForest version 4.6-10). In the first analysis, C3d/C3 was included as the dependent variable for the regression type random forest test. In the second analysis, the disease status was defined as the classifier for a classification type of random forest. For both analyses the number of predictors sampled for splitting at each node was set to two. All other options were left at default setting.

5.5 SUPPLEMENTARY DATA

Table S1. Association between AMD and SNP genotypes in the *CFH*, *CFB* and *C3* genes

SNP	cDNA change	Protein change	Genotype	N		P-value	OR	95% C.I.for EXP(B)	
				AMD	Control			Lower	Upper
CFH rs800292	c.134G>A	p.V62I	GG	1101	801	$7.4*10^{-11}$			
			GA	445	539	0.015	0.63	0.435	0.913
			AA*	62	77	$2.4*10^{-11}$	0.564	0.477	0.667
CFB rs4151667	c.26T>A	p.L9H	TT*	1511	1291	0.02			
			TA	99	127	0.904	0.828	0.038	17.888
			AA	1	1	0.005	0.654	0.486	0.881
CFB rs641153	c.95G>A	p.R32Q	GG*	1436	1195	$3*10^{-4}$			
			GA	171	217	0.387	0.545	0.138	2.152
			AA	4	7	$7.9*10^{-5}$	0.623	0.492	0.788
C3 rs2230199	c.304G>C	p.R102G	CC*	911	901	$2.4*10^{-6}$			
			CG	570	471	0.046	1.183	1.003	1.396
			GG	117	47	$7.8*10^{-7}$	2.59	1.775	3.777

Analyses were performed by logistic regression analysis. The genotypes marked with * are the ancestral variants. Variables entered in the model: *CFH* rs800292, *CFB* rs4151667, *CFB* rs641153, *C3* rs2230199, age and gender. Bonferroni corrected threshold for statistical significance is p<0.004.

Table S2. Genotype combination frequency for the novel complotype

***CFB* (rs4151667) - *CFB* (rs641153) - *CFH* (rs800292)**	**Control**		**AMD**		**Total**
	n	**%**	**n**	**%**	
AA - GG - GG	1	0.1	1	0.1	2
TA - GA - AA	0	0.0	2	0.1	2
TT - AA – GA	1	0.1	1	0.1	2
TA - GA - GA	5	0.4	1	0.1	6
TT - AA - GG	6	0.4	3	0.2	9
TA - GG - AA	7	0.5	3	0.2	10
TA - GA - GG	10	0.7	5	0.3	15
TT - GA - AA	11	0.8	9	0.6	20
TA - GG - GA	47	3.4	23	1.4	70
TA - GG - GG	55	3.9	65	4.1	120
TT - GG - AA	59	4.2	48	3.0	107
TT - GA - GA	74	5.3	47	2.9	121
TT - GA - GG	112	8.0	106	6.6	218
TT - GG - GA	406	29.0	370	23.1	776
TT - GG - GG	607	43.3	916	57.3	1523
Total	1401	100	1600	100	3001

Table S3. Differences in mean complement activation levels between genotype combinations

CFB **(rs4151667) -** ***CFB*** **(rs641153) -** ***CFH*** **(rs800292)**		**Mean Difference (I-J)**	**Std. Error**	**P-value**
TA - GG - GA	TA - GG - GG	-0.024	0.026	1
	TT - GA - GA	-0.041	0.027	1
	TT - GA - GG	-0.085*	0.024	0.011
	TT - GG - AA	-0.06	0.027	0.524
	TT - GG - GA	-0.079*	0.022	0.006
	TT - GG - GG	-0.100*	0.021	$6.1*10^{-5}$
TA - GG - GG	TA - GG - GA	0.024	0.026	1
	TT - GA - GA	-0.017	0.024	1
	TT - GA - GG	-0.061	0.021	0.074
	TT - GG - AA	-0.036	0.023	1
	TT - GG - GA	-0.055*	0.018	0.043
	TT - GG - GG	-0.076*	0.017	$1.9*10^{-4}$
TT - GA - GA	TA - GG - GA	0.041	0.027	1
	TA - GG - GG	0.017	0.024	1
	TT - GA - GG	-0.044	0.021	0.843
	TT - GG - AA	-0.019	0.024	1
	TT - GG - GA	-0.038	0.018	0.826
	TT - GG - GG	-0.059*	0.018	0.019
TT - GA - GG	TA - GG - GA	0.085*	0.024	0.011
	TA - GG - GG	0.061	0.021	0.074
	TT - GA - GA	0.044	0.021	0.843
	TT - GG - AA	0.025	0.021	1
	TT - GG - GA	0.006	0.014	1
	TT - GG - GG	-0.015	0.014	1
TT - GG - AA	TA - GG - GA	0.06	0.027	0.524
	TA - GG - GG	0.036	0.023	1
	TT - GA - GA	0.019	0.024	1
	TT - GA - GG	-0.025	0.021	1
	TT - GG - GA	-0.019	0.018	1
	TT - GG - GG	-0.04	0.018	0.464
TT - GG - GA	TA - GG - GA	0.079*	0.022	0.006
	TA - GG - GG	0.055*	0.018	0.043
	TT - GA - GA	0.038	0.018	0.826
	TT - GA - GG	-0.006	0.014	1
	TT - GG - AA	0.019	0.018	1
	TT - GG - GG	-0.021	0.008	0.215
TT - GG - GG	TA - GG - GA	0.100*	0.021	$6.1*10^{-5}$
	TA - GG - GG	0.076*	0.017	$1.9*10^{-4}$
	TT - GA - GA	0.059*	0.018	0.0189
	TT - GA - GG	0.015	0.014	1
	TT - GG - AA	0.04	0.018	0.464
	TT - GG - GA	0.021	0.008	0.215

*The mean difference is significant at the 0.05 level. All p-values were adjusted for multiple comparisons: Bonferroni. The general linear model was corrected for age, gender, BMI and disease status.

Table S4. Amino acid conservation for *CFH* (rs800292, p.V62I) - *CFB* (rs4151667, p.L9H) - *CFB* (rs641153, p.R32Q) - *C3* (rs2230199, p.R102G)

Species	CFH p.V62I	CFB p.L9H	CFB p.R32Q	C3 p.R102G
Human	V	L	R	R
Chimp	I	L	Q	R
Mouse	I	L	R	G
Dog	I	L	A	G
Cat		L	G	G
Caw		L	G	G

5.5.1 Statistical Syntax used for the models built in SPSS and the R script used to run the Random forest analyses:

SPSS syntax for the statistical models:

UNIANOVA Log_C3d_C3 BY Gender Disease_status Complotype_SNP2_SNP3_SNP4 WITH Age_Blooddate Q14_BMI

/METHOD=SSTYPE(3)

/INTERCEPT=INCLUDE

/EMMEANS=TABLES(Complotype_SNP2_SNP3_SNP4)

WITH(Age_Blooddate=MEAN Q14_BMI=MEAN) COMPARE

ADJ(BONFERRONI)

/PRINT=ETASQ PARAMETER

/CRITERIA=ALPHA(.05)

/DESIGN=Gender Disease_status Complotype_SNP2_SNP3_SNP4 Age_Blooddate Q14_BMI.

LOGISTIC REGRESSION VARIABLES Disease_status

/METHOD=ENTER Complotype_SNP2_SNP3_SNP4 Age_Blooddate Gender

/CONTRAST (Complotype_SNP2_SNP3_SNP4)=Indicator

/CLASSPLOT

/PRINT=CI(95)

/CRITERIA=PIN(0.05) POUT(0.10) ITERATE(20) CUT(0.5).

The R script used for the random forest analyses

```
library(randomForest)
setwd("")
data <- read.table("file.txt", header=T)
View(data)
Combi3_4_SNPs <- data[,(2:11)]
attach(Combi3_4_SNPs)
set.seed(4)
complotype.rf <- randomForest(Log_C3d_C3 ~ ., data=Combi3_4_SNPs, mtry=2, importance=TRUE)
print(complotype.rf)
round(importance(complotype.rf), 2)

library(randomForest)
setwd("")
data <- read.table("file2.txt", header=T)
View(data)
selected_colums <- data[, (2:11)]
names(selected_colums)
attach(selected_colums)
sink("results_randomeForest_on_disease_status.txt")
set.seed(4)
complotype_on_AMD.rf <- randomForest(Disease_status ~ ., data=selected_colums, importance=TRUE, proximity=TRUE)
print(complotype_on_AMD.rf)
round(importance(complotype_on_AMD.rf), 2)
```

5.6 REFERENCES

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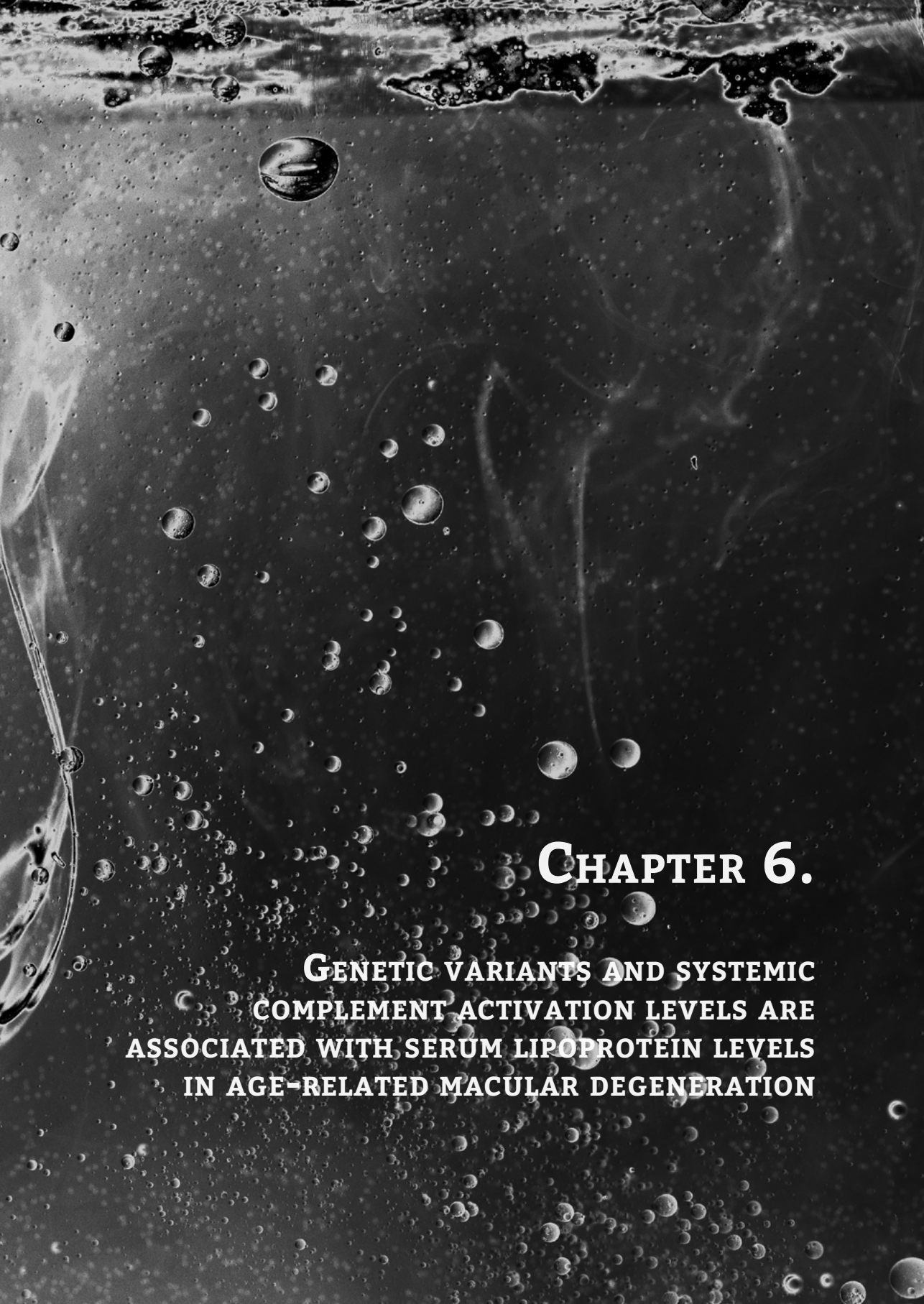

Chapter 6.

Genetic variants and systemic complement activation levels are associated with serum lipoprotein levels in age-related macular degeneration

Adapted from

Genetic variants and systemic complement activation levels are associated with serum lipoprotein levels in age-related macular degeneration

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ABSTRACT

Purpose Genetic variants in genes encoding components of lipid metabolism have been associated with AMD. The aims of this study were to evaluate the relation of these genetic variants with serum lipid levels in AMD in a large case-control cohort (n = 3070) and to test for correlations between lipids and complement activation.

Methods Single nucleotide polymorphisms (SNPs) in eight lipid metabolism genes, previously described to be associated with AMD, were genotyped and tested for their association in our case-control cohort. Serum apolipoprotein B (ApoB), apolipoprotein AI (Apo-AI), cholesterol, triglycerides (TG), high-density lipoprotein–cholesterol (HDLC), and complement activation levels (C3d/C3) were measured and tested for association with AMD. Non-HDL cholesterol and LDL were inferred based on the measurements of the other lipids and lipoproteins. General linear models and $\chi 2$ tests were used to evaluate the relation of SNPs and lipids/lipoproteins to the disease as well as their interrelations.

Results Significant genotypic associations with AMD were observed for SNPs in *CETP*, *APOE*, and *FADS1*. The serum levels of Apo-AI and HDLC were significantly higher in patients compared with controls. Triglycerides (TG) levels were lower in AMD compared with controls. A cumulative effect was observed for *APOE* and *CETP* genotypes on HDLC and Apo-AI levels. Complement activation levels correlated positively with HDLC and Apo-AI, and negatively with TG. Both the lipids/lipoproteins and the complement activation levels associate independently to AMD.

Conclusions This study bridges the gap between genetic associations and physiological lipid levels in AMD. Additionally, the observed correlations between complement activation and lipid levels link two major systems that previously were always assessed independently.

6.1 INTRODUCTION

Age-related macular degeneration (AMD) is a multifactorial, progressive disease and a leading cause of blindness in the elderly population.[1,2] The strong genetic underpinnings of AMD based on genome-wide association studies (GWAS) broadly point toward the involvement of three systems in the pathogenesis of AMD: the complement system, lipid metabolism, and the extracellular matrix.[3] Investigating the pathways identified by genetic associations has proven to be a fruitful research strategy in the past. A higher rate of systemic complement activation levels was demonstrated in patients compared with controls,[4,5] bringing systemic physiological consequences in line with the genetic associations. For the second major system involved in AMD, the lipid metabolism, such congruency is not immediately apparent.

Lipids, due to their insoluble nature, are transported through the circulation by lipoproteins.[6] Two major lipoproteins of this process are low-density lipoprotein (LDL) and high-density lipoprotein (HDL).[7] Low-density lipoprotein is responsible for transporting cholesterol from the liver to the periphery, while HDL transports peripheral cholesterol back to the liver in a process called reverse cholesterol transport (RCT).[8]

In a recent GWAS on AMD, three genes involved in the lipoprotein transport system (*CETP*, *APOE*, and *LIPC*) reached genome-wide significance.[3] In addition, two earlier association studies also reported associations for *LPL, FADS1*, and *ABCA1*.[9,10] All of the proteins encoded by these genes, are either enzymes, coenzymes, or transporters within the lipid metabolism. Thus, ample genetic evidence exists for the involvement of lipid metabolism in the etiology of AMD.

In the pathology of AMD, aberrant lipid homeostasis has also been observed. Particularly, approximately 40% of drusen composition (one of the major hallmarks of AMD) is made up of esterified cholesterol, unesterified cholesterol, and phosphatidylcholine.[11] However, it has proven to be challenging to attribute risk scores for the development of AMD to systemic measurements of HDL or LDL. Studies directly investigating the levels of HDL/HDLC and LDL report conflicting results, with some presenting higher levels of HDL/HDLC in AMD patients compared with controls,[12–18] whereas others describe the opposite.[19–21] Yet other reports, including those that combined multiple of the previous studies, did not observe any significant differences between patients and controls.[1,22–26]

It is important to clarify the role of HDL and other lipids/lipoproteins in AMD and their relation to the established AMD-associated lipid genes, as this may shed more light on the pathogenesis of AMD. Such insights potentially could lead to targeted and more efficient approaches toward treatment regimens for AMD. Therefore, the primary aim of this study was to investigate the relation of single nucleotide polymorphisms (SNPs) genotypes in the AMD-associated lipid genes and serum lipid levels in AMD in a large case-control cohort (N = 3070). The secondary aim was to determine if there is a correlation between the previously described complement activation[4] and lipid levels.

6.2 MATERIALS AND METHODS

6.2.1 Study Population

From the European Genetic Database (EUGENDA, in the public domain, www.eugenda.org), 3070 participants above the age of 50 years were included in the study. The study was performed in accordance with the tenets of the Declaration of Helsinki and the Medical Research Involving Human Subjects Act (WMO), and was approved by the local ethics committee of the University Hospitals in Cologne and Nijmegen. Written informed consent was obtained from all participants.

Age-related macular degeneration and control status were assigned by multimodal image grading that included stereo fundus photographs, fluorescein angiograms, and spectral-domain optical coherence tomograms. The grading was performed according to the standard protocol of the Cologne Image Reading Center (CIRCL) by certified graders (TR, LE). The classification of AMD was performed as described previously.[27]

Demographic data and nongenetic parameters including smoking status (current/past/never), regular alcohol intake (current/past/never), body mass index (BMI), exercise/physical activity (never, almost never, 1–2 times a week, 3 or more times a week), and daily fat consumption (more than 35 g oil per day: Yes/No) were obtained by standardized interviewer-assisted questionnaires.

6.2.2 Serum Measurements and Genetic Analysis

Serum samples were used for the various lipid and systemic complement measurements. Serum was obtained by a standard coagulation/centrifugation protocol, after which the samples were stored at −80°C within 1 hour after collection. Serum levels of

apolipoprotein B (Apo-B), apolipoprotein AI (Apo-AI), total cholesterol, triglycerides (TG), and HDL-cholesterol (HDLC) were measured in all patients and controls using standard procedures by a clinical chemistry laboratory (Architect Analyzer; Abbott Diagnostics Hoofddorp, The Netherlands). Non-HDL cholesterol (NHDL) was calculated by subtracting HDLC from total cholesterol; and low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald formula.[28]

Complement component C3 and the activation fragment C3d were measured in serum samples as previously described[29] and C3d/C3 was calculated as a measure of complement activation.

Genomic DNA was extracted from peripheral blood samples using standard procedures. Eight SNPs in the *LIPC, CETP, APOE, FADS1, LPL*, and *ABCA1* genes (see Supplementary Table S1) were genotyped using the KASPar SNP Genotyping System by LGC Genomics.

6.2.3 Statistical Analysis

All calculations were performed using SPSS software version 20.0 (IBM Software and Systems, Armonk, NY, USA). Associations between lipid levels, SNP genotypes and disease status were analyzed using general linear models with each lipid, in turn, set as the dependent variable. The first model was built to find the association between lipids and disease status, the model was corrected for all possible confounders (see Table 3). The second model was built to find the association between lipids and SNP's, again the model corrected for all possible confounders (see Table 4). In literature smoking status, alcohol intake, BMI, and dietary fat intake are reported to significantly influence lipid and lipoprotein levels,[30–34] for this reason they were selected as correction factors for the models to eliminate any possible confounding. Additionally, age, sex, and exercise/physical activity were significantly different between patients and controls, thus they were also added as correction factors.

In order to assess the cumulative effect of *CETP* and *APOE* SNPs on lipid/lipoprotein levels, a new variable was created that had all nine possible genotype combinations (see Table 4). The association with the lipid/lipoprotein levels was tested in a general linear model also corrected for age, sex, BMI, smoking status, alcohol intake, exercise/physical activity, daily fat consumption, and disease status. The significance threshold was corrected for multiple testing, P values less than or equal to 0.006 (0.05/8 associations per experiment) were considered statistically significant for the associations to AMD

of both SNP genotypes and serum lipid levels. For the associations of the SNPs and serum lipid levels we have corrected for 12 associations (4 genetic variables against 3 lipid/lipoprotein levels). The P values less than or equal to 0.004 (0.05/12 studied associations) were considered significant.

Associations between AMD phenotype and genotypes were evaluated using cross tabulation, P values were calculated with Pearson $\chi 2$ and odds ratios were generated using logistic regression. Pearson correlations were used to investigate the relationship between lipids and complement activation levels.

All power calculations were performed using CaTS - Power Calculator v0.0.2 (Center for Statistical Genomics, University of Michigan, Ann Arbor, MI, USA) as previously described.[35] For the calculation we assumed a multiplicative model, a disease prevalence of 10% and a significance level of 0.006 (0.05/8 SNPs). The disease allele frequency and genotype relative risk were extracted from the papers that first described the associations (Supplementary Table S1).

6.3 RESULTS

Summary of the demographics for the subjects included in the present study are shown in Table 1.

Eight SNPs in genes of the lipid metabolism, previously shown to be associated with AMD, were selected from literature (see Supplementary Table S1). Out of the eight SNPs that were analyzed, genotypes in *CETP* (rs3764261; P = 0.002), *APOE* (rs4420638; P = 0.005), and *FADS1* (rs174547; P = 0.005), were significantly associated with AMD after correcting for multiple testing (P < 0.006). A summary of all associations and genotype frequencies are presented in Table 2.

Mean serum levels of Apo-B, Apo-AI, total cholesterol, HDLC, LDL, NHDL, and triglycerides of AMD patients compared to controls are presented in Table 3. After adjusting for age, sex, BMI, smoking status, alcohol intake, exercise/physical activity, and daily fat consumption, AMD patients had significantly higher Apo-AI (P = 0.002) and HDLC levels ($P = 4.4 \times 10^{-5}$) compared with controls. In contrast, patients had significantly lower serum levels of TG ($P = 1.9 \times 10^{-4}$) compared with

Table 1. Baseline characteristics of study subjects.

	EUGENDA	
	AMD (n=1491)	Control (n = 1579)
Female sex, %	60	58
Mean age ± SD, y	74.7 ± 8.4	69 ± 7.6
Age range, y	50-101	50-100
Mean BMI ± SD	25.82 ± 3.8	25.79 ± 3.9
Smoking status, %		
current	10.2	8.5
past	47.6	47.5
never	42.2	44
Regular alcohol intake, %		
current	57.6	56.4
past	5	4.2
never	37.4	39.4
Exercise/physical activity, %		
never	24	20.1
almost never	14.6	6.8
1-2 times a week	44.2	47
3 or more times a week	17.2	26.1
Daily fat consumption, %		
yes	28.8	30.6
no	71.2	69.4

Significant differences between the two groups were only for age ($p=1.98x10^{-114}$), sex ($p=0.043$) and exercise/physical activity ($p=9.9x10^{-25}$)

controls. Significant positive correlations were observed between Apo-AI and HDLC, and negative correlations between HDLC and TG (Supplementary Figure S1). The association between Apo-AI and AMD was independent of HDLC and TG. Similarly, the association of TG with AMD was independent of HDLC and Apo-AI. When correcting for Apo-AI and TG, the association of HDLC with AMD was negated, which is in line with the correlation of HDLC with Apo-AI and TG levels. No significant associations were found for any of the other measurements.

Table 2. Association of genotypes in lipid metabolism genes with AMD.

SNP	Gene	P*	Genotype	Status (%)		OR	95% C.I.
				Control	AMD		
rs493258	*LIPC*	0.388	TT	21.1	19.6		
			CT	49.0	48.6	1.07	0.89-1.27
			CC	29.9	31.8	1.14	0.94-1.38
rs10468017	*LIPC*	0.161	TT	8.2	7.7		
			CT	41.9	39.1	0.99	0.76-1.28
			CC	49.9	53.2	1.13	0.88-1.46
rs3764261	*CETP*	0.002	GG	47.8	42.3		
			GT	41.2	44.2	1.21	1.05-1.40
			TT	10.9	13.5	1.40	1.12-1.74
rs2075650	*APOE*	0.043	GG	1.6	1.4		
			GA	23.5	20.1	0.96	0.54-1.69
			AA	74.9	78.5	1.18	0.68-2.05
rs4420638	*APOE*	0.005	GG	3.0	1.9		
			GA	28.6	25.3	1.41	0.89-2.21
			AA	68.3	72.8	1.70	1.09-2.65
rs12678919	*LPL*	0.579	AA	80.7	79.3		
			AG	18.1	19.4	1.10	0.92-1.30
			GG	1.3	1.2	0.99	0.54-1.81
rs174547	*FADS1*	0.005	CC	11.8	8.5		
			CT	43.1	43.8	1.41	1.12-1.79
			TT	45.1	47.7	1.47	1.16-1.86
rs3758294	*ABCA1*	0.982	TT	63.1	62.9		
			TC	32.6	32.8	1.03	0.73-1.45
			CC	4.3	4.2	1.02	0.73-1.42

* after correction for multiple testing significance is reached at P ≤ 0.006

Stratifying for the different AMD stages revealed significant associations only with the intermediate AMD stage. The observed effect directions were similar to the comparison of all AMD stages versus controls, with only Apo-AI, HDLC, and TG being significantly associated with intermediate AMD after correction for multiple testing (Supplementary Table S2).

The SNPs that were significantly associated with AMD in the EUGENDA cohort, were analyzed for association with the lipid/lipoprotein levels that significantly differed between patients and controls. Only *APOE* (rs4420638) and *CETP* (rs3764261) genotypes displayed significant associations with Apo-AI and HDLC serum levels. *APOE* (rs4420638) genotypes were moderately associated with TG levels (P = 0.026), however the association did not remain significant after correcting for multiple testing. A summary of the results is presented in Table 4.

Table 3. Association of mean serum lipid/lipoprotein levels with AMD*.

Lipid	Status	Mean	SE	P
Apo-B, mg/l	Control	954.98	9.50	0.788
	AMD	957.44	9.41	
Apo-AI, mg/l	Control	1615.76	11.43	0.002
	AMD	1649.35	11.32	
Total Cholesterol, mM	Control	5.60	0.05	0.155
	AMD	5.66	0.05	
HDLC, mM	Control	1.40	0.01	$4.6x10^{-5}$
	AMD	1.45	0.01	
LDL, mM	Control	3.83	0.04	0.739
	AMD	3.85	0.04	
NHDL, mM	Control	4.20	0.05	0.819
	AMD	4.21	0.04	
Triglycerides, mM	Control	1.76	0.03	$1.9x10^{-4}$
	AMD	1.65	0.03	

* General linear models were built with each lipid/lipoprotein as the dependent variable. The models tested for the association to disease status and were all corrected for age, sex, BMI, smoking status, alcohol intake, exercise/physical activity and daily fat consumption. Threshold for statistical significance p<0.006.

Because both *CETP* and *APOE* SNP genotypes were significantly associated with Apo-AI and HDLC serum levels, the cumulative effect of carrying multiple risk genotypes on the lipid levels was investigated. Mean levels for each genotype combination of *CETP* and *APOE* are displayed in Table 4 and visualized in Figure 1. In both cases, carriers of double high-risk genotypes for *CETP* and *APOE* showed significantly elevated levels of Apo-AI and HDLC compared with low-risk genotype carriers.

To exclude the possibility that the associations of HDLC and Apo-AI with AMD were mainly a consequence of the underlying genetic associations of *CETP* and *APOE* that drive HDLC and Apo-AI levels, all lipid analyses were corrected for *CETP* and *APOE* genotypes and all the other genotyped SNPs. After doing so, HDLC and Apo-AI remained significantly associated with AMD, independent of the genotypes ($P = 1.4 \times 10^{-4}$ and 0.003, respectively).

Table 4. Association of AMD SNPs with serum lipid/lipoprotein levels.*

		Lipid/lipoprotein					
		Apo-AI, mg/L		HDLC, mM		TG, mM	
SNP	**Genotype**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**
CETP rs3764261	GG	1601.6	12.1	1.37	0.01	1.71	0.03
	GT	1645.9	12.0	1.46	0.01	1.71	0.03
	TT[a]	1704.6	17.8	1.54	0.02	1.73	0.05
	Sig.	$2.8x10^{-9}$		$6.50x10^{-20}$		0.950	
APOE rs4420638	GG	1555.7	35.9	1,32	0.04	1.95	0.09
	GA	1612.6	13.8	1.42	0.02	1.73	0.04
	AA[a]	1643.6	11.0	1.44	0.01	1.71	0.03
	Sig.	0.003		0.012		0.026	
FADS1 rs174547	CC	1620.4	18.6	1.44	0.02	1.75	0.05
	TC	1632.8	12.1	1.43	0.01	1.74	0.03
	TT[a]	1638.5	12.0	1.43	0.01	1.69	0.03
	Sig.	0.595		0.832		0.137	
CETP/APOE	GG/GG	1531.3	48.8	1.26	0.06	2.1	0.13
	GG/GA	1561.4	18.0	1.33	0.02	1.7	0.05
	GG/AA	1619.0	13.1	1.39	0.02	1.7	0.04
	GT/GG	1590.9	54.6	1.41	0.06	1.8	0.14
	GT/GA	1651.7	18.2	1.48	0.02	1.7	0.05
	GT/AA	1644.2	13.0	1.45	0.02	1.7	0.03
	TT/GG	1530.6	123.3	1.29	0.14	2.2	0.36
	TT/GA	1660.3	29.7	1.51	0.03	1.8	0.08
	TT/AA	1725.3	20.7	1.56	0.02	1.7	0.05
	Sig.	$1.3x10^{-9}$		$4.4x10^{-19}$		0.118	

*The model was corrected for age, sex, BMI, smoking status, alcohol intake, exercise/physical activity, daily fat consumption and disease status. Threshold for statistical significance p<0.004. Sig., significance.

a. Risk allele for AMD in our cohort

Finally, because serum complement activation levels were previously shown to be associated to AMD,[4] we tested whether a relation exists between lipid levels and complement activation levels (C3d/C3 ratio). This analysis revealed significant positive correlations between Apo-AI, HDLC, and complement activation and a significant negative correlation for TG (Figure 2). All P values were less than 1.9×10^{-9}. A general linear model corrected for disease status and other variables confirmed the association of C3d/C3 to lipids/lipoproteins ($P = < 1.9 \times 10^{-9}$) and revealed that the association to disease status is independent of lipid levels ($P = 9 \times 10^{-6}$).

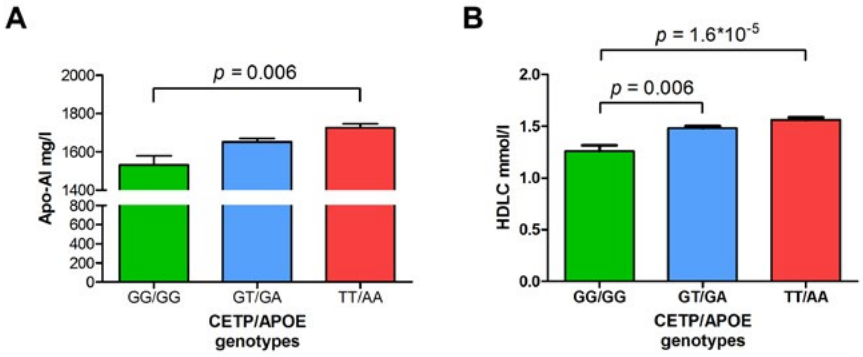

Figure 1. Mean lipid levels with standard error bars for *CETP/APOE* homozygous high-risk genotypes, heterozygous and homozygous low-risk genotypes. All P values are Bonferroni-corrected. (A) Levels for Apo-AI; (B) levels for HDLC.

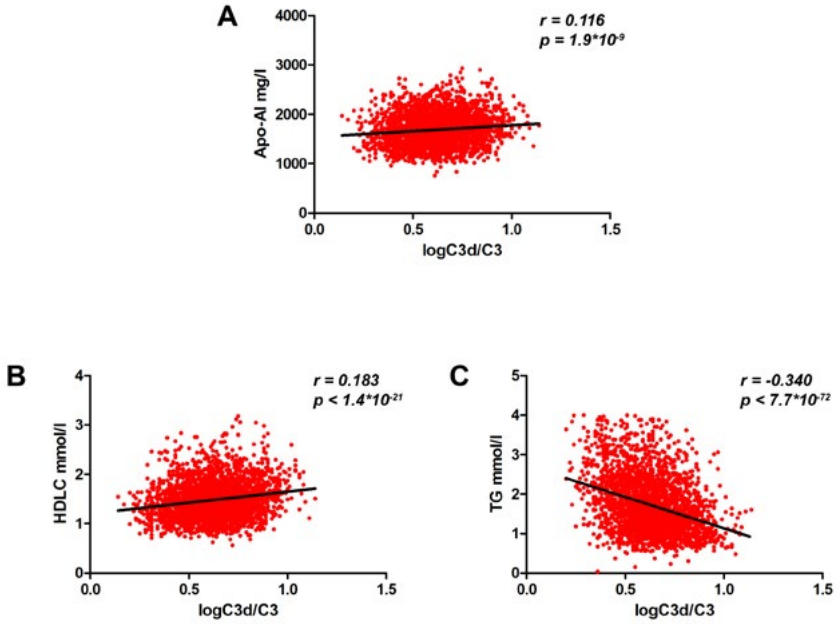

Figure 2. Scatterplots showing the correlations of lipid levels with complement activation levels represented by the log transformed ratio of C3d/C3. Direction of the correlations is indicated by the black regression line. (A) Positive correlation of Apo-AI and logC3d/C3; (B) positive correlation of HDLC with logC3d/C3; (C) negative correlation of TG and logC3d/C3.

6.4 DISCUSSION

The genetic analyses from the present case-control study confirm previously described associations for *CETP* (rs3764261), *APOE* (rs4420638), and *FADS1* (rs174547) with AMD. However, no associations were observed for *APOE* (rs2075650), *LIPC* (rs493258 and rs10468017), *LPL* (rs12678919), and *ABCA1* (rs3758294). The SNPs were selected from recent large GWAS[3,9,10,36,37] (see Supplementary Table S1). For the SNPs in *ABCA1* (rs1883025) and *LIPC* (rs493258 and rs10468017) our study was underpowered with 52%, 59%, and 53% chance of detection, respectively. Therefore, we cannot exclude the possibility that these SNPs may be associated to AMD in a larger cohort. On the other hand, for *LPL* (rs12678919) this study had 77% detection power, suggesting that not all genetic associations may be reliably replicated between different populations.

In this study, we observed significant differences in the serum levels of Apo-AI, HDLC, and TG of AMD patients compared with controls. Triglycerides were significantly lower, while Apo-AI and HDLC were significantly higher in patients compared with controls. No statistically significant associations with AMD were detected for any of the other measured lipids/lipoproteins.

In the literature, there are inconsistent associations of AMD with serum lipid levels. Comparing the mean lipid/lipoprotein levels observed in the present study with values previously reported (Supplementary Table S3), is challenging since the measurements were performed differently across the various reports, different correction factors were applied, and different populations were studied. All of these factors can influence the mean levels, making it difficult to pinpoint the cause of the different study outcomes. However, if we compare the main effects, our findings of high HDLC levels in patients compared with controls are consistent with several previous studies.[12–18] The positive association of HDLC with only the intermediate AMD stage confirms the finding reported by Cougnard-Grégoire et al.[12] On the other hand, other publications have reported inverse or no association between HDLC and AMD.[19–23,25,26] When results were pooled in a meta-analysis, no associations have been detected.[1,24,38] To our knowledge, for Apo-AI this is the first large study to report a positive association with AMD, and for TG other studies reported opposite or no associations with AMD.[12,20,26] The reasons for these inconsistencies are not fully understood, however in a recent publication high levels of HDLC were associated with risk for AMD only after a stringent multivariate correction.[12] Because our study, and others,[39–41] show a clear effect of genotype on lipid levels, correcting for these

genotypes may improve the insight into the associations of lipid levels with AMD and the direction of their effect. This is especially important because our study, although appropriately powered, had failed to detect associations with *LPL* (rs12678919), suggesting that population- or cohort-specific genetic substructures may account partly for the observed inconsistencies. Another reason could be related to sample size, which in some studies might not be large enough to allow for the necessary adjustments and still have sufficient power to detect significant associations. In our cohort, higher levels of Apo-AI and HDLC were associated with risk genotypes in *CETP* (rs3764261; TT) and *APOE* (rs4420638; AA). A cumulative effect was observed for these two SNPs, with a risk-allele dose dependent increase in both HDLC and Apo-AI serum levels (Figure 1). The *CETP* and *APOE* loci have previously been linked to lipid metabolism in cardiovascular studies.[40] In the context of AMD, few studies have looked into the relation of AMD lipid SNPs and serum lipid levels. Our results for *CETP* were consistent with a recent report from the Alienor study.[12] Another study observed that in individuals carrying the *LPL* (rs12678919) GG genotype, TG levels were significantly lower and HDLC levels were significantly higher.[42] Moreover, one study reported that the *LIPC* (rs10468017) T allele was associated with higher levels of HDLC.[43] Our study does not describe an association of *LPL* and *LIPC* genotypes with lipid levels, because no significant difference between patients and controls was observed.

CETP encodes for cholesterol ester transfer protein (CETP), which promotes the transfer of excess cholesterol ester (CE) to the liver through the RCT pathway.[44] Several studies have shown that lower CETP activity leads to higher HDLC levels.[41,45,46] *APOE* encodes for apolipoprotein E (ApoE), which plays a major role in the metabolism of cholesterol and TG by mediating the clearance of chylomicrons and very low-density–lipoprotein (VLDL) from the bloodstream.[47,48] ApoE has been described to have a direct relation with CETP by enhancing the CE and TG transfer between VLDL and HDL in a CETP-dependent manner.[49] Despite the direct impact of ApoE and CETP on HDLC metabolism, understanding how the risk genotypes of the studied SNPs could have the cumulative HDLC raising effect is not directly obvious, mainly because both rs3764261 and rs4420638 are located in intergenic regions. One possibility may be a consequence of an effect on *CETP* expression levels that was reported for rs3764261.[50]

Traditionally, rs4420638 is reported as an *APOE* SNP, because it is considered a proxy for rs429358, one of the two coding variants that determine the *APOE* isoforms (ε2, ε3, and ε4) reported to attenuate binding affinity to the (LDL)-receptor,[51] and thus affect the entire cholesterol metabolism. However, the r2 value for the linkage disequilibrium

of these two SNPs is 0.63,[3] indicating that there is not a complete coinheritance. Also, its genomic position is closer to the *APOC1* gene, a potent inhibitor of CETP activity,[52] thus we cannot exclude the possibility of rs4420638 for being a proxy for a regulatory variant of *APOC1* instead.

Understanding the local involvement of lipid and lipoprotein systems at AMD disease sites in the eye is made difficult by the lack of information regarding eye specific function of these molecules. Nevertheless, if we focus on HDLC metabolism, clues can be found. First, key components of the RCT pathway for which the main player is HDLC,[53] are expressed in the retina.[54–56] In addition, during the normal aging process, an accumulation of Apo-B of unusual composition takes place in the Bruch's membrane, forming a precursor of basal linear deposit, called the "lipid wall."[57] Moreover, the macromolecular conductivity of the Bruch's membrane reduces 10-fold between the first and ninth decades of life, which is significant because lipoproteins need to cross the Bruch's membrane in order to mediate lipid efflux from the RPE.[58,59] Furthermore, in vitro, HDL has been observed to mediate efflux of photoreceptor outer segment lipids from the basal surfaces of RPE cells.[60] Finally, a retention of cholesterol in drusen, the major lesions of AMD, has been reported.[61]

Besides the involvement of HDL in lipid and lipoprotein transport, this system has recently been implicated in immune function.[62] Recent proteomic analyses revealed several types of HDL particles containing complement system components C4a, C4b, C9, and vitronectin[63,64] in healthy subjects, and C3 in patients with coronary artery disease.[63] In our study, we offer support for this emerging concept by demonstrating a significant correlation between HDLC and complement system activation, although it remains to be determined whether the effect is direct or indirect.

One possible limitation of our study may be that the lipid/lipoprotein levels were not overnight fasted blood measurements, which could induce possible artifacts for certain lipids like the TG. However, the fact that HDLC and Apo-AI levels are not severely affected by food intake,[65] and the great number of participants in this study, negate this potential drawback.

In our study, the genetic and environmental factors explain 31%, 84%, and 27% of the Apo-AI, HDLC, and TG variation, respectively. Our findings indicate that other factors must be associated with them, which might relate to the AMD disease pathogenesis.

In conclusion, the results of our study indicate that patients with high risk *CETP/APOE* genotypes and high HDLC levels have higher risk of developing AMD, suggesting that they could potentially benefit from HDLC lowering regimens. Further studies are needed to investigate the role of HDL subfractions and the observed correlation of HDLC with complement activation in the disease pathogenesis of AMD.

6.5 SUPPLEMENTARY DATA

Table S1. Lipid associated SNPs and the papers they were selected from:

SNP	Gene	Reference
rs493258	*LIPC*	9
rs10468017	*LIPC*	9
rs3764261	*CETP*	3, 9, 10
rs2075650	*APOE*	64, 65
rs4420638	*APOE*	3
rs12678919	*LPL*	9, 10
rs174547	*FADS1*	9
rs3758294	*ABCA1*	9, 10

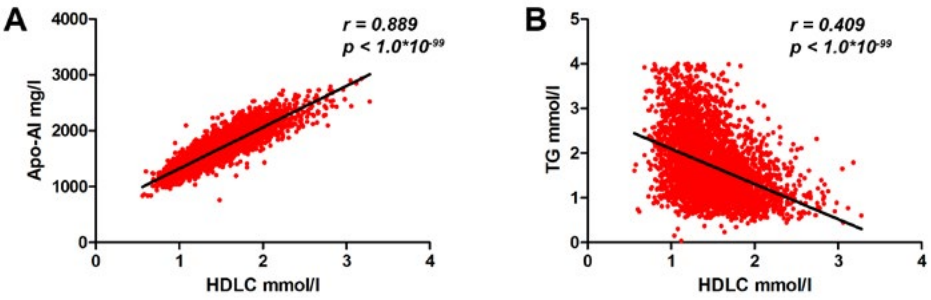

Figure S1. Correlations of Apo-AI and TG with HDLC in the EUGENDA cohort. The direction of the correlations is indicated by the black regression line. A. positive correlation between Apo-AI and HDLC levels; B. negative correlation between TG and HDLC levels.

Table S2. Association of mean serum lipid/lipoprotein levels with AMD subgroups*. The AMD subgroups are: early AMD, intermediate AMD, advanced AMD - geographic atrophy (GA), advanced AMD with choroidal neovascularization (CNV) and advanced AMD with both GA and CNV (mixed).

Disease status		Apo-B			Total Cholesterol			LDL			NHDL		
		Mean Difference (Control-AMD)	Std. Error	P	Mean Difference (Control-AMD)	Std. Error	P	Mean Difference (Control-AMD)	Std. Error	P	Mean Difference (Control-AMD)	Std. Error	P
Control	Early	0.0003	0.006	0.964	0.019	0.069	0.784	0.018	0.063	0.775	0.037	0.067	0.574
	Intermediate	0.013	0.007	0.062	0.023	0.074	0.753	0.079	0.068	0.245	0.122	0.071	0.088
	Advanced GA	0.008	0.012	0.509	0.063	0.133	0.633	0.067	0.12	0.577	0.074	0.127	0.559
	Advanced CNV	-0.011	0.006	0.053	-0.162	0.064	0.012	-0.11	0.059	0.063	-0.118	0.062	0.056
	Advanced mixed	-0.003	0.018	0.865	-0.078	0.193	0.687	0.039	0.176	0.825	-0.047	0.185	0.799

Disease status		Apo-AI			HDLC			Triglycerides		
		Mean Difference (Control-AMD)	Std. Error	P	Mean Difference (Control-AMD)	Std. Error	P	Mean Difference (Control-AMD)	Std. Error	P
Control	Early	6.669	17.3	0.7	-0.019	0.02	0.35	0.077	0.045	0.088
	Intermediate	-73.938	18.828	$8.8*10^{-5}$	-0.106	0.022	$1.7*10^{-6}$	0.239	0.048	$7.6*10^{-7}$
	Advanced GA	-15.471	33.109	0.64	-0.02	0.039	0.601	0.108	0.086	0.208
	Advanced CNV	-47.942	16.027	0.003	-0.052	0.019	0.005	0.094	0.042	0.026
	Advanced mixed	-31.589	48.051	0.511	-0.04	0.056	0.478	-0.182	0.126	0.148

*The model was corrected for age, gender, BMI, smoking status, alcohol intake, exercise/physical activity, daily fat consumption and all relevant SNPs. Threshold for statistical significance p<0.001 (0.05/40 associations).

Table S3. Mean lipid/lipoprotein values compared across various studies. Reference 24 reported the results from three different studies: Beaver Dam Eye Study (BDES), Blue Mountains Eye Study (BMES) and Rotterdam Study (RS). All values are displayed in the original reported measurement unit.

Lipid/lipoprotein	Disease status	EUGENDA	Reference					
			12	24 (BDES)	24 (BMES)	24 (RS)	20	26
Apo-B	Control	954.98 mg/l					121.62 mg/dl	
	AMD	957.44					164.66	
Apo-AI	Control	1615.76 mg/l					160.32 mg/dl	
	AMD	1649.35					128.9	
Total Cholesterol	Control	5.60 mmol/l	5.78 mM	232.7 mg/dl	234.2 mg/dl	258.2 mg/dl	190.86 mg/dl	174.4 mg/l
	AMD	5.66	5.81				224.36	209.9
HDLC	Control	1.40 mmol/l	1.56 mM	53.2 mg/dl	55.8 mg/dl	52.3 mg/dl	49.2 mg/dl	45.12 mg/l
	AMD	1.45	1.66				38.68	44.12
LDL	Control	3.83 mmol/l	3.66 mM				125.2 mg/dl	95.46 mg/l
	AMD	3.85	3.62				159.02	121.93
NHDL	Control	4.20 mmol/l		179.6 mg/dl	178.4 mg/dl	205.9 mg/dl		
	AMD	4.21						
Tryglicerides	Control	1.76 mmol/l	1.24 mM				101.72 mg/dl	110.56 mg/l
	AMD	1.65	1.17				120.92	141.46

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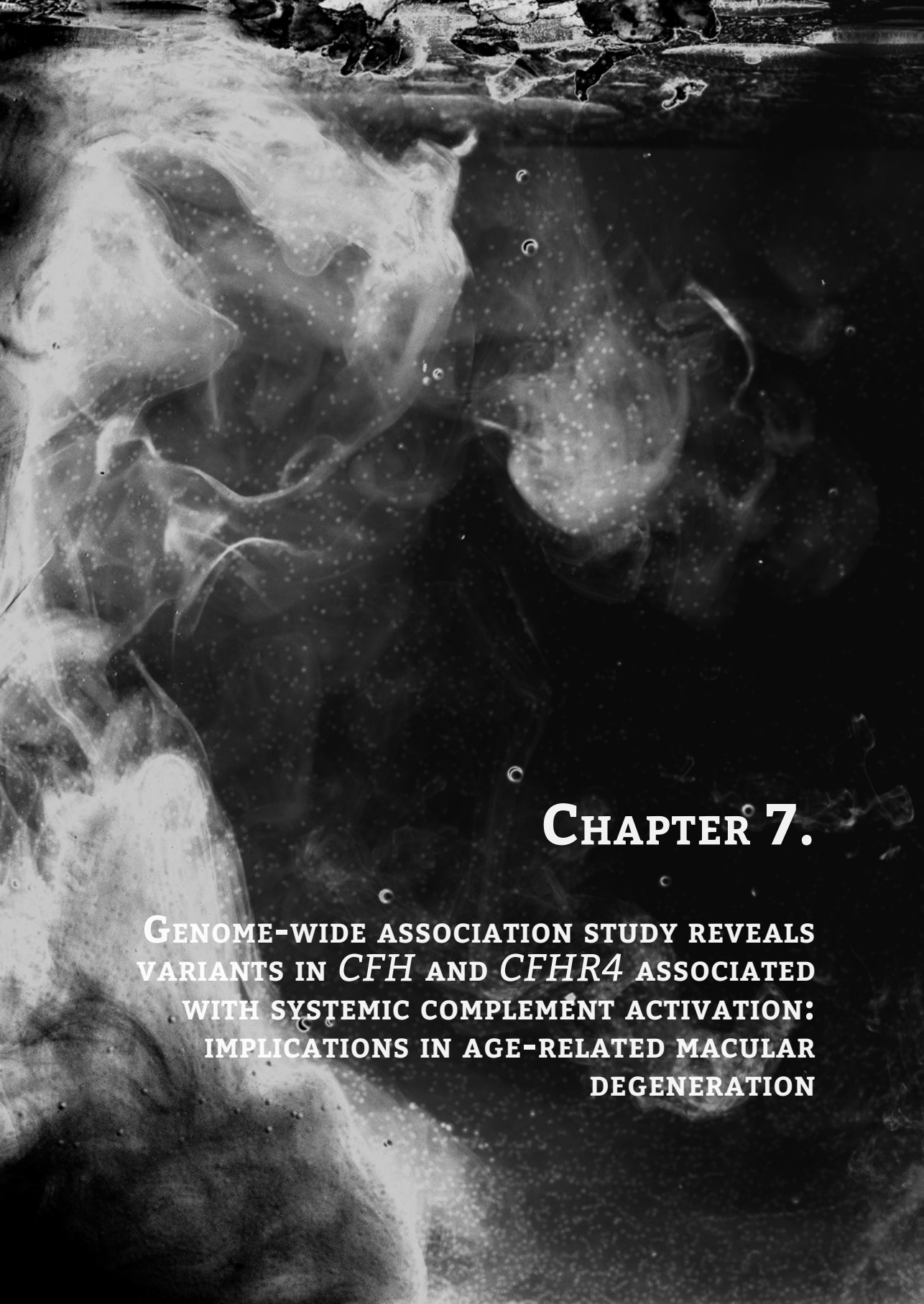

Chapter 7.

Genome-wide association study reveals variants in *CFH* and *CFHR4* associated with systemic complement activation: implications in age-related macular degeneration

Adapted from

Genome-wide association study reveals variants in *CFH* and *CFHR4* associated with systemic complement activation: implications in age-related macular degeneration

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ABSTRACT

Purpose To identify genetic variants associated with complement activation, which may help to select age-related macular degeneration (AMD) patients for complement-inhibiting therapies.

Design Genome-wide association study (GWAS) followed by replication and meta-analysis.

Participants AMD patients and controls (n = 2245).

Methods A GWAS on serum C3d-to-C3 ratio was performed in 1548 AMD patients and controls. For replication and meta-analysis, 697 additional individuals were genotyped. A model for complement activation including genetic and non-genetic factors was built, and the variance explained was estimated. Haplotype analysis was performed for 8 SNPs across the *CFH/CFHR* locus. Association with AMD was performed for the variants and haplotypes found to influence complement activation.

Main Outcome Measures Normalized C3d/C3 ratio as a measure of systemic complement activation.

Results Complement activation was associated independently with rs3753396 located in *CFH* (Pdiscovery = 1.09×10^{-15}; Pmeta = 3.66×10^{-21}; β = 0.141; standard error [SE] = 0.015) and rs6685931 located in *CFHR4* (Pdiscovery = 8.18×10^{-7}; Pmeta = 6.32×10^{-8}; β = 0.054; SE = 0.010). A model including age, AMD disease status, body mass index, triglycerides, rs3753396, rs6685931, and previously identified SNPs explained 18.7% of the variability in complement activation. Haplotype analysis revealed 3 haplotypes (H1–2 and H6 containing rs6685931 and H3 containing rs3753396) associated with complement activation. Haplotypes H3 and H6 conferred stronger effects on complement activation compared with the single variants (P = 2.53×10^{-14}; β = 0.183; SE = 0.024; and P = 4.28×10^{-4}; β = 0.144; SE = 0.041; respectively). Association analyses with AMD revealed that SNP rs6685931 and haplotype H1–2 containing rs6685931 were associated with a risk for AMD development, whereas SNP rs3753396 and haplotypes H3 and H6 were not.

Conclusions The SNP rs3753396 in *CFH* and SNP rs6685931 in *CFHR4* are associated with systemic complement activation levels. The SNP rs6685931 in *CFHR4* and its linked haplotype H1–2 also conferred a risk for AMD development, and therefore could be used to identify AMD patients who would benefit most from complement-inhibiting therapies.

7.1 INTRODUCTION

The complement system is an integral part of our innate immunity. Its best known physiologic functions are host defense against foreign intruders and homeostasis maintenance.[1] It consists of more than 30 plasma proteins and cellular components that interact in proteolytic cascades for an efficient and rapid activation leading to inflammation, opsonization, and targeted cytolysis.[2] The complement system can be activated by 3 different pathways: the classical pathway, the lectin pathway, and the alternative pathway (AP). The classical pathway is activated by antibody–antigen complexes and the lectin pathway is activated by lectin or ficolin binding to carbohydrates, both present on the surfaces of pathogens. In contrast, the AP is activated constitutively at a low level in a process known as tick-over.[3]

All 3 pathways lead to the formation of complement component 3 (C3) convertases that catalyze a proteolytic cleavage of complement C3 into the potent anaphylatoxin C3a, and C3b, an opsonization molecule that can be further cleaved into C3d. Complement component 3b also can bind the cleaved form of factor B (Bb) to form the AP C3 convertase (C3bBb) that will cleave more C3, initiating an amplification loop. Downstream in the cascade, complement component 5 convertases are formed, initiating the terminal pathway with the subsequent formation of additional activation products as well as the membrane-attack complex that is responsible for cytolysis.[4] The complement system can be amplified rapidly, and therefore several inhibitory proteins such as complement factor H (FH) and complement factor I are in place regulating complement activity.[4]

Deregulation and deficiencies of the complement system have been reported to be associated with numerous inflammatory, autoimmune, neurodegenerative, and infectious disorders.[5] A prime example of a multifactorial disease associated with a deregulation of the complement system is age-related macular degeneration (AMD). Age-related macular degeneration is characterized by a progressive degeneration of the central retina and is responsible for most cases of vision loss in the elderly with a pooled prevalence of 8.9%.[6,7] Age-related macular degeneration constitutes a major health problem as by 2020, the number of people affected by a form of this disease is projected to be 196 million, rising to 288 million by 2040.[8] Several lines of evidence point toward an overactivation of the complement system in AMD, mainly through a dysregulation of the AP. Multiple genetic variants in or near complement genes (*CFH, C3, CFI, C2/CFB* locus, and *C9*) have been associated strongly with AMD.[9,10]

Moreover, complement components have been described in drusen, the hallmark of the disease,[11,12,13,14] and complement activation fragments in plasma or serum such as Ba, C3a, C3d, and component C5a have been found to be elevated significantly in AMD patients compared with controls.[15,16,17,18,19,20,21] Currently, there is no treatment available for most forms of AMD, nor is there an effective means to halt AMD progression. Therefore, therapies for AMD, as well as for other diseases involving complement deregulation, are being developed aiming to inhibit or lower complement activation.[22,23,24]

Systemic complement activation levels demonstrate considerable variation among individuals.[16,17,18,19,20] As a consequence, patients who have higher levels of complement activation may benefit more than others from the upcoming therapies. A better understanding of the factors that influence complement activation would facilitate the selection of the most suitable patients for complement-inhibiting therapies. Genetic markers are robust biomarkers that could be included in prediction models for complement activation. Several studies have previously evaluated the effect of genetic variation on complement activity; however, these studies were restricted to a limited number of single nucleotide polymorphisms (SNPs).[16,17,18,19,21,25]

The aim of this study was to perform the first genome-wide association study (GWAS) on systemic complement activation levels. Identification of genetic variants explaining complement activation levels will contribute to a better understanding of the molecular mechanisms of complement-related diseases, will pinpoint potential drug targets, and will facilitate the selection of patients for complement-inhibiting therapies.

7.2 MATERIALS AND METHODS

7.2.1 Study Population

This study included 2245 participants from the European Genetic Database (www.eugenda.org). The European Genetic Database is a multicenter database for the clinical and molecular analysis of AMD collected at the Radboud University medical center, Nijmegen, The Netherlands, and at the University Hospital of Cologne, Cologne, Germany. The study participants were separated into 2 cohorts: a discovery cohort comprising 1548 individuals and a replication cohort of 697 individuals.

The study was performed in accordance with the tenets of the Declaration of Helsinki (seventh revision) and the Medical Research Involving Human Subjects Act. Approval of the local ethics committee of both University hospitals was obtained, and written informed consent was acquired from all participants. All the individuals included in the study agreed to the performed serum measurements and genotyping. All participants were of European descent and older than 50 years. Age-related macular degeneration and control status were assigned by multimodal image grading according to the standard protocol of the Cologne Image Reading Center by certified graders. Age, sex, height, and weight measurements were obtained by standardized interviewer-assisted questionnaires.

7.2.2 Serum Complement and Lipid Measurements

Serum was obtained by a standard coagulation and centrifugation protocol, and within 1 hour after collection, the samples were stored at −80°C. Triglycerides and high-density lipoprotein cholesterol were measured using standard procedures by a clinical chemistry laboratory (Architect Analyzer; Abbott Diagnostics, Hoofddorp, The Netherlands). Complement component 3 was assessed by radial immunodiffusion (or Mancini method) using monospecific polyclonal rabbit antisera, and C3d was measured by rocket electrophoresis, as previously described.[21] Complement component 3d is a fragment of C3 generated upon activation of the system, and therefore a direct measurement of complement turnover.[4] Moreover, C3d has the longest half-life of all C3 split products.[26] The C3d-to-C3 ratio is a sensitive way of assessing the activation of the complement system independently of the baseline individual C3 concentration.[27,28,29] The C3d-to-C3 ratio has been described previously to be a robust biomarker for complement activation in AMD studies.[19] The different measurements were performed for all samples in a single assay.

7.2.3 Genotyping

Genomic DNA was extracted from peripheral blood samples using standard procedures. The discovery cohort was genotyped with a custom-designed HumanCoreExome array by Illumina (Illumina Inc., San Diego, CA) within the International AMD Genetics Consortium. All the details regarding the design of the array, annotation, imputation, and quality control of the genotypic data have been described previously.[9]

Imputed lead variants in GWAS peaks that reached significance, rs6685931 and rs3130572, were confirmed by polymerase chain reaction and Sanger sequencing.

The SNP rs6685931 was evaluated in 12 individuals representing the 3 genotypes, and a 100% of concordance with the imputed genotypes was achieved. The SNP rs3130572 (chromosome 6) was located in a highly repetitive region and specific primers could not be designed; therefore, this SNP was excluded from further analysis. In the replication cohort, *CFH* rs3753396 and *CFHR4* rs6685931 were genotyped using competitive allele-specific polymerase chain reaction assays according to the manufacturer instructions (KASP Genotyping Chemistry; LGC, Hoddesdon, UK).

7.2.4 Statistical Analysis

Natural log transformation was applied to normalize the skewed distribution of C3d/C3 measurements. A general linear model for ln(C3d/C3) including as independent variables the environmental factors collected was used to determine potential confounders. The R2 and adjusted R2 statistics were estimated for the model. Additionally, the R2 statistic was estimated for each of the independent factors individually, performing separate models. Analyses were carried out using SPSS software version 20.0 (IBM Software and Systems, Armonk, NY).

A power calculation for the GWAS was performed using the Genetic power calculator.[30] Association tests in the GWAS and replication analyses were performed by means of a linear Wald test from EPACTS software (http://genome.sph.umich.edu/wiki/EPACTS) using allele dosages. Linear regression models adjusted for age, sex, body mass index (BMI), triglycerides, clinic site, and the first 2 ancestry principal components were used. Manhattan and Q-Q plots were generated using the 'qqman' R package (version 0.1.2; R Foundation for Statistical Computing, Vienna, Austria). The regional plots for chromosome 1 were generated using LocusZoom.[31] Meta-analysis of fixed effects based on effect size estimates and standard errors was performed using METAL software (version 2-11-03-25).[32]

Evaluation of an interaction between the identified SNPs and clinic or AMD status was performed including an interaction parameter on the general linear model and assessing nominal significance. Comparisons of systemic complement activation levels between the genotype groups were performed using a general linear model adjusted for age, BMI, triglycerides, and clinic sites including both the discovery and the replication cohorts. SPSS software version 20.0 (IBM Software and Systems, Armonk, NY) was used for these analyses.

To estimate how much of the variation in systemic complement activation could be explained by the identified factors, general linear models for systemic complement activation were performed using SPSS software version 20.0. Only the 1548 individuals from the discovery cohort were included to accommodate the *CFH* rs800292 and *C2* rs9332739 SNPs, which were not analyzed in the replication cohort. The adjusted R2 statistic was estimated for the models.

Haplotype analysis was carried out for the 1548 patients genotyped with exome-arrays using the haplo.glm function of the R library 'haplo.stats' (version 1.7.7). Analysis was performed based on a general linear model adjusted for age, sex, BMI, triglycerides, clinic site, and the first 2 ancestry principal components.

Single-variant and haplotype association analyses with AMD were performed for the 1548 individuals of the discovery cohort. Single-variant analyses were performed using a Firth bias-corrected likelihood-ratio test with EPACTS software. Haplotype analyses were based on chi-squared tests including haplotypes with a predicted probability of 0.75 or more using SPSS software version 20.0.

Risk scores for AMD-associated variants were calculated as a sum of the number of AMD risk-increasing alleles. Two risk scores were calculated: the first risk score included the 52 AMD-associated variants described in Fritsche et al,[9] and the second risk score included the 19 variants located in or near complement genes of these 52. The variants included in the complement risk score were: rs10922109, rs570618, rs121913059, rs148553336, rs187328863, rs61818925, rs35292876, and rs191281603 from the *CFH* locus; rs10033900 and rs141853578 from the *CFI* locus; rs62358361 from the *C9* locus; rs116503776, rs144629244, rs114254831, and rs181705462 from the *C2/CFB/SKIV2L* locus; rs11080055 from the *TMEM97/VTN* locus; and rs2230199, rs147859257, and rs12019136 from the *C3* locus. The risk scores were included in linear models for ln(C3d/C3) that included age, BMI, triglycerides, and clinic site as covariates, and the effect of the risk score was estimated. The 1548 individuals from the discovery phase, genotyped with the HumanCoreExome array, were included in these analyses. Figures including graphs were generated using Graphpad Prism version 5.03 (GraphPad Software, La Jolla, CA).

7.3 RESULTS

7.3.1 Characteristics of the Study Cohorts

We evaluated the association of genetic variants with systemic complement activation levels through a GWAS in a discovery cohort of 1548 individuals, followed by replication in an independent cohort of 697 individuals. For both cohorts, demographics and information about AMD disease status, BMI, triglycerides, and high-density lipoprotein cholesterol was collected (Table 1).

7.3.2 Genome-Wide Association Study Identifies 2 Independent Signals at the *CFH/CFHR* Locus to Be Associated with Systemic Complement Activation

We carried out a GWAS of normalized C3d/C3 levels as a measure of systemic complement activation. After quality control, a total of 1548 individuals and 9 972 920 variants were included in the analysis. The study had more than 80% of power to detect common variants (minor allele frequency ≥5%), explaining ≥2.6% or more of variance in complement activation levels.

Table 1. Demographics and other characteristics of the discovery and replication cohorts

	Discovery cohort (n=1,548)	Replication cohort (n=697)
Complement activation ln(c3d/c3), mean (SD)	1.459 (0.407)	1.464 (0.398)
Age, mean (SD)	73.2 (7.8)	73.3 (7.7)
Female sex (%)	60	58.8
AMD disease status, control (%)	53.7	37.4
BMI (kg/m2), median (quartiles)	25 (23 – 28)	25 (23 – 28)
Triglycerides (mmol/l), median (quartiles)	1.620 (1.170 - 2.220)	1.620 (1.165 - 2.210)
HDL cholesterol (mmol/l), mean (SD)	1.489 (0.377)	1.478 (0.403)
Clinic site-Radboud university medical center, %	53.5	63

AMD = age-related macular degeneration; BMI = body mass index, HDL = high-density lipoprotein, SD = standard deviation.

Higher complement activation levels were associated independently with older age, AMD disease status, lower BMI, and lower triglyceride levels as previously described.[21, 33] Differences also were observed between the sample collection clinics (Table S1). Therefore, these factors were included as covariates in all consecutive analyses.

A total of 280 variants reached genome-wide significance (Manhattan plot, Figure 1A; QQplot, Figure S1; λGC = 0.999). All variants, except for one, were located on chromosome 1 at the *CFH/CFHR* locus (chromosome 1, 196.643.724–197.061.086). The only variant outside of this locus was located on chromosome 6 near the *PSORS1C1* gene, but could not be verified by Sanger sequencing. The SNP rs3753396 (c.2016A→G, p.Gln672Gln) located in exon 14 of the complement factor H (*CFH*) gene showed the strongest association with complement activation levels ($P = 1.09 \times 10^{-15}$; β = 0.145; standard error [SE], 0.018; Table 2; locus zoom depicted in Figure 1B).

Conditional analysis on the lead SNP revealed a second independent signal with a P value close to genome-wide significance for which the strongest associated variant was rs6685931. This SNP was also located at the *CFH/CFHR* locus, specifically in intron 1 (c.59–4315T→C) of the complement factor H related 4 (*CFHR4*) gene ($P = 8.18 \times 10^{-7}$; β = 0.068; SE, 0.014; Table 2; locus zoom depicted in Figure 1C).

Variants shown to be associated with complement activation fragments in previous studies were extracted from the GWAS results.[17,18] The SNP rs800292 in *CFH* and the 2 SNPs in linkage disequilibrium rs4151667 in *CFB* and rs9332739 in *C2* were associated nominally with systemic complement activation levels in the current study, showing the same direction of the effect. The SNP rs2230199 in *C3* and the SNP rs10490924 in *ARMS2* could not be replicated (Table S2).

7.3.3 Replication in an Independent Cohort Confirms the Effect of rs3753396 in CFH and rs6685931 in CFHR4 on Systemic Complement Activation

Replication analysis of rs3753396 in *CFH* and rs6685931 in *CFHR4* in an independent cohort of 697 study participants confirmed both variants to be associated significantly with systemic complement activation levels (rs3753396: $P = 1.39 \times 10^{-6}$; β = 0.131; SE, 0.027; and rs6685931: $P = 8.62 \times 10^{-3}$; β = 0.038; SE, 0.014; Table 2). Subsequent meta-analysis showed associations for both rs3753396 ($P = 3.66 \times 10^{-21}$; β = 0.141; SE, 0.015) and rs6685931 ($P = 6.32 \times 10^{-8}$; β = 0.054; SE, 0.010), confirming that 2 independent signals at the *CFH/CFHR* locus are associated with higher complement activation levels (Table 2). Sensitivity analyses adjusting for AMD disease status showed comparable results (Table S3), and neither an interaction between clinic site and the identified SNPs (Prs3753396 × clinic = 0.436; Prs6685931 × clinic = 0.676), nor an interaction between AMD status and the identified SNPs (Prs3753396 × AMD status = 0.557; Prs6685931 × AMD status = 0.658) was detected.

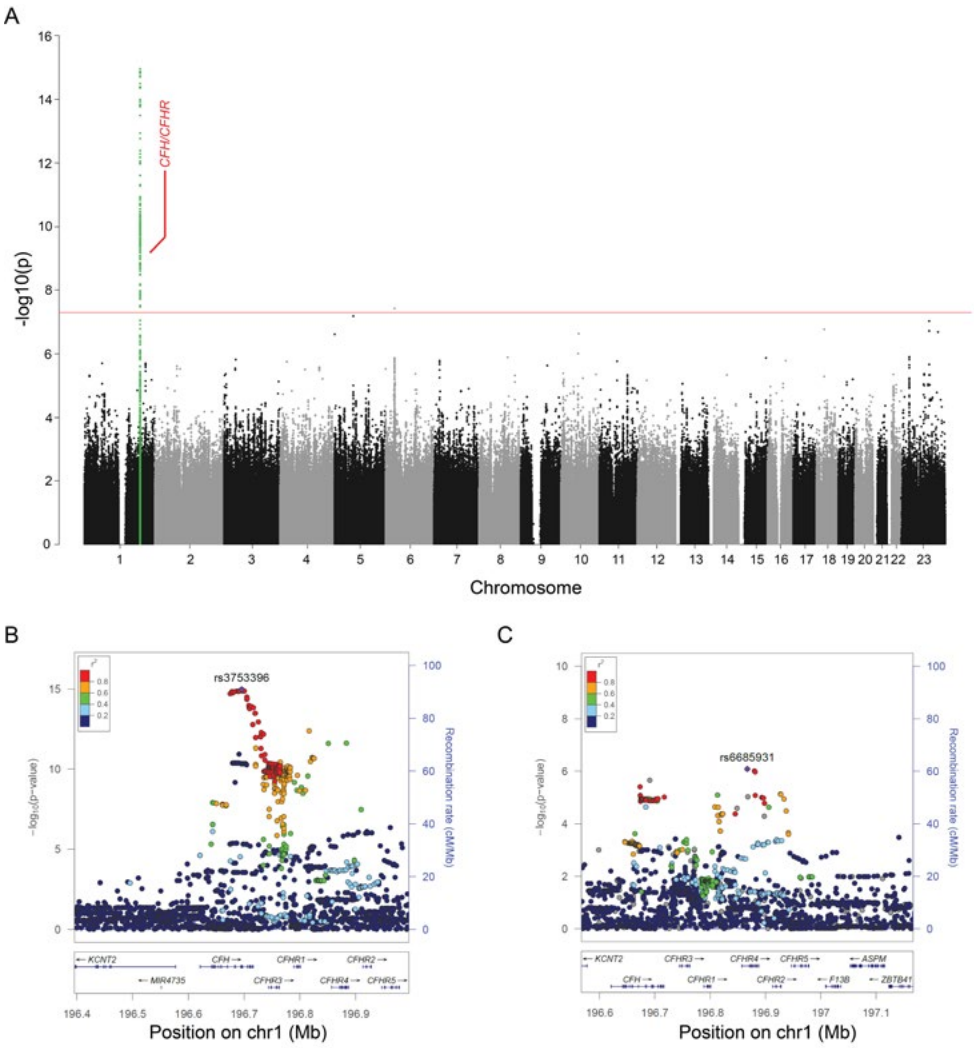

Figure 1. Graphs showing that the genome-wide association study identified 2 independent signals at the *CFH/CFHR* locus associated with systemic complement activation levels. A, Manhattan plot illustrating the P values of each individual single nucleotide polymorphism (SNP) tested for association with systemic complement activation. The red horizontal line indicates the threshold considered for genome-wide significance ($P = 5 \times 10^{-8}$). B, Locus zoom plot showing a detailed view of the chromosome 1 signal. The lead SNP rs3753396 is located in the *CFH* gene. The SNPs are colored based on their linkage disequilibrium estimate (r2) to the lead SNP. C, Locus zoom plot showing a detailed view of the signal on chromosome 1 (chr1) after conditioning the association analysis for rs3753396. Here, the lead SNP rs6685931 is located in the *CFHR4* gene. The SNPs are colored based on their linkage disequilibrium estimate (r2) to the lead SNP.

Table 2. Meta-analysis of discovery and replication cohorts identifies two signals at the *CFH/CFHR* locus associated with systemic complement activation levels

				Discovery cohort (n=1,548)			Replication cohort (n=697)§			Meta-analysis (n=2,245)¶	
Lead variant (MA)	**Imputation quality (Rsq)***	**Chr.: Position†**	**Gene‡**	**MAF**	**β (SE)**	***P*-value**	**MAF**	**β (SE)**	***P*-value**	**β (SE)**	***P*-value**
rs3753396 (G)	—	1:196,695,742	*CFH*	0.168	0.145 (0.018)	1.091×10^{-15}	0.147	0.131 (0.027)	1.390×10^{-6}	0.141 (0.015)	3.664×10^{-21}
rs6685931 (C)	0.99	1:196,867,233	*CFHR4*	0.439	0.068 (0.014)	8.184×10^{-7}	0.493	0.038 (0.014)	8.620×10^{-3}	0.054 (0.010)	6.320×10^{-8}

MA=Minor allele, Chr=chromosome, MAF=Minor allele frequency, SE=Standard error.

*Not applicable for genotyped variants (—).
†Chromosome and chromosomal positions described according to the reference sequence database of the National Center for Biotechnology Information (NCBI RefSeq) hg19 human genome.
‡Closest gene to the lead variant.
§Replication cohort for rs6685931 consisted of 686 individuals.
¶Meta-analysis for rs6685931 was performed in a total of 2234 individuals.

Next, mean complement activation levels in the genotype groups of rs373396 and rs6685931 were analyzed. For rs3753396 in *CFH*, the heterozygous AG genotype group showed higher complement activation levels compared with the reference AA genotype group ($P = 6.23 \times 10^{-18}$; $\beta = 0.152$; SE, 0.018), and for the homozygous GG group, these levels were even higher ($P = 2.39 \times 10^{-7}$; $\beta = 0.267$; SE, 0.052; Figure 2A). In the case of rs6685931 in *CFHR4*, a similar effect was observed: the heterozygous TC genotype group had higher complement activation levels than the reference TT genotype ($P = 10^{-3}$; $\beta = 0.063$; SE, 0.019) and for the homozygous CC group, the levels were even higher ($P = 3.62 \times 10^{-7}$; $\beta = 0.118$; SE, 0.023; Figure 2B). Analysis of the cumulative effect of both SNPs showed that the main effect on systemic complement activation levels is driven by rs3753396 in *CFH*, and rs6685931 in *CFHR4* introduces additional variation to the rs3753396 genotypes (Figure 2C).

7.3.4 A Model of Genetic and Nongenetic Variables Explains 18.7% of the Variability in Complement Activation

General linear models were built to determine how much of the variation could be explained by factors found to be associated with systemic complement activation. A model including only non-genetic factors (age, AMD disease status, BMI, and triglycerides) explained 12.6% of the variability in systemic complement activation. With the addition of SNP rs3753396 to the model, 16.3% of the variability could be explained, and by including SNP rs6685931, a total of 17.3% was explained. We additionally incorporated SNPs associated with complement activation fragments in a previous study that replicated in our GWAS: rs800292 in *CFH* and rs9332739 in *C2*.[18] Only rs9332739 remained associated independently with systemic complement activation levels, and the variance explained by the model rose to 18.7% (adjusted R2; Table 3).

7.3.5 Haplotypes across the *CFH/CFHR* Locus Show Stronger Effects on Systemic Complement Activation Levels Compared with Individual Variants

To assess whether more variants at the *CFH/CFHR* locus influence systemic complement activation and to determine the cumulative effect of several variants on the same haplotype, we evaluated the effect of distinct haplotypes across the *CFH/CFHR* locus on systemic complement activation. Haplotypes previously described for AMD already included rs3753396, the lead variant associated in the GWAS,[34] and were expanded by adding rs668593, the second independent signal. In total, 7 SNPs

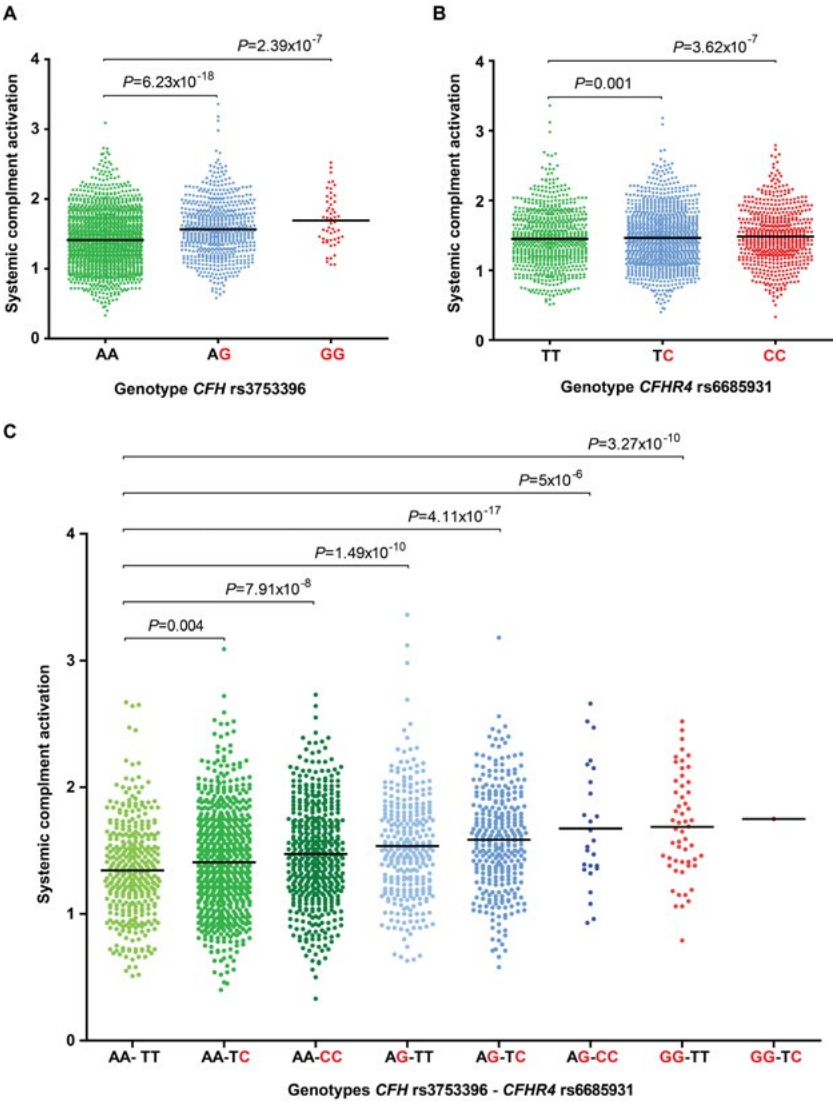

Figure 2. Graphs showing systemic complement activation levels stratified by rs3753396 and rs6685931 genotypes: rs6685931 introduced additional variation on the main effect of rs3753396. The y-axes represent the ln-transformed complement C3d/C3 ratio as a measure of systemic complement activation. Horizontal bars indicate the mean values for each genotype group. The complement-raising alleles for both single nucleotide polymorphisms are indicated in red. Association analyses included the 2245 individuals from the discovery and the replication cohorts. A, Distribution of complement activation levels for each genotype of rs3753396 in CFH. B, Distribution of complement activation levels for each genotype of rs6685931 in CFHR4. P values were calculated adjusting the model for rs3753396. C, Distribution of complement activation levels over the genotype combinations of rs3753396 in *CFH* and rs6685931 in *CFHR4*.

Table 3. A model of genetic and non-genetic variables explains 18.7% of the variability in systemic complement activation

		β	SE (β)	*P*-value
***CFH* rs3753396**	**AG**	0.196	0.023	$8.772x10^{-17}$
	GG	0.330	0.066	$6.461x10^{-7}$
***CFHR4* rs6685931**	**TC**	0.070	0.024	0.003
	CC	0.125	0.033	$1.620x10^{-4}$
***CFH* rs800292**	**GA**	-0.011	0.023	0.639
	AA	0.027	0.046	0.555
***C2* rs9332739**	**GC**	-0.185	0.034	$4.674x10^{-8}$
	CC	0.168	0.213	0.431
Age (years)		0.004	0.001	0.005
Disease status (AMD)		0.035	0.020	0.089
BMI (kg/m2)		-0.012	0.003	$2x10^{-6}$
Triglycerides (mmol/l)		-0.131	0.011	$1.177x10^{-33}$

AMD = age-related macular degeneration; BMI = body mass index.

R2 = 0.193 (adjusted R2 = 0.187). The model included the 1548 individuals from the discovery phase.

across the *CFH/CFHR* locus yielded 9 different haplotypes with a predicted population frequency higher than 1% (Table 4; Table S4).

Haplotype association analyses with age-related macular degeneration were performed for the 1548 individuals in the discovery cohort. Haplotypes are coded as in Hageman et al.[34] If 2 different subhaplotypes based on the extra allele in single nucleotide polymorphism rs6685931 were found, the Hageman haplotypes were recoded as 1 or 2. Alleles associated with higher complement levels are underlined. The reference haplotype was set to the most common haplotype not carrying any complement-raising allele for rs3753396 or rs6685931.

Association with systemic complement activation levels revealed haplotypes with stronger effects on complement activation compared with the single SNPs identified in the GWAS. Haplotypes H1–2, H3, and H6 were associated with higher systemic complement activation levels. Haplotype H3 carrying the complement-raising allele of rs3753396 (G) had a stronger effect on complement activation levels ($P = 2.53 \times 10^{-14}$; β = 0.183; SE, 0.024) compared with the complement-raising allele of rs3753396 in the single variant analysis (β = 0.141; SE, 0.015). Haplotypes H1–2 and H6 both carried the complement-raising allele for rs6685931 (C). Haplotype H6 showed a stronger effect on complement activation levels ($P = 4.82 \times 10^{-4}$; β = 0.144; SE, 0.041) compared with the single variant analysis for rs6685931 (β = 0.054; SE, 0.010; Table 4; Table S4).

Table 4. Association of haplotypes across the *CFH/CFHR* locus with systemic complement activation levels

Haplotype	*CFH* rs3753396 and *CFHR4* rs6685931 Alleles	Haplotype Frequency	β	Standard Error (β)	*P* value
H2	A-T	0.18	Reference	Reference	Reference
H1-2	A-C	0.36	0.062	0.019	1.148×10^{-3}
H3	G-T	0.14	0.183	0.024	2.531×10^{-14}
H4	A-T	0.10	0.013	0.026	0.607
H5	A-T	0.04	-0.058	0.038	0.128
H1-1	A-T	0.03	-0.053	0.043	0.218
H6	A-C	0.03	0.144	0.041	4.823×10^{-4}
H7	A-C	0.03	0.060	0.046	0.192
H8	A-T	0.03	-0.007	0.048	0.890

Haplotype association analyses with age-related macular degeneration were performed for the 1548 individuals in the discovery cohort. Haplotypes are coded as in Hageman et al.34 If 2 different subhaplotypes based on the extra allele in single nucleotide polymorphism rs6685931 were found, the Hageman haplotypes were recoded as 1 or 2. Alleles associated with higher complement levels are underlined. The reference haplotype was set to the most common haplotype not carrying any complement-raising allele for rs3753396 or rs6685931.

7.3.6 The Single Nucleotide Polymorphism rs6685931 in *CFHR4* and Haplotype H1–2 Confer a Risk for Age-Related Macular Degeneration

To identify genetic biomarkers that are relevant in the context of disease, we explored whether the SNPs and haplotypes associated with systemic complement activation levels also associate with AMD. The SNP rs3753396 in *CFH* was not associated with AMD (P = 0.76). In contrast, the complement-raising allele of rs6685931 in *CFHR4* (C) was associated with an increased risk for AMD ($P = 5.89 \times 10^{-12}$; odds ratio = 1.631; 95% confidence interval, 1.489–1.772; Table 5). These results are in concordance with the largest GWAS on AMD reported to date (rs3753396, $P = 3 \times 10^{-3}$; rs6685931, $P = 1.02 \times 10^{-495}$; odds ratio >1).[9]

In agreement with the single variant analysis of *CFH* rs3753396, the haplotype H3 that gave the highest risk for higher systemic complement activation was not associated with AMD (P = 0.80). Haplotype H6 carries the *CFHR4* rs6685931 complement-raising allele (C), but did not reach significance in the association with AMD (P = 0.14); however, the frequency of H6 was relatively low (3%). Haplotype H1–2, the most

common haplotype carrying the *CFHR4* rs6685931 complement-raising allele (C), showed a strong risk-conferring association with AMD ($P = 1.38 \times 10^{-12}$; odds ratio = 1.318; 95% confidence interval, 1.223–1.420; Table 5; Figure 3).

Finally, we determined whether other AMD-associated variants are associated with systemic complement activation levels. For this purpose, we extracted the 52 AMD-associated variants reported in the largest AMD study performed so far from the GWAS on complement activation levels.[9] However, no variants outside of the *CFH/CFHR* locus were found to be associated with systemic complement activation levels at the genome-wide significance level, or at a significance level of $P < 0.05/52 = 0.001$ (Table S5). Interestingly, a risk score based on the 52 AMD risk-conferring alleles was associated with higher levels of complement activation ($P = 0.043$; $\beta = 0.004$; $SE(\beta) = 0.002$). A similar risk score including only the variants located in or near complement genes was associated more strongly with higher levels of complement activation ($P = 0.022$; $\beta = 0.009$; $SE(\beta) = 0.004$). This complement risk score included 3 nominally associated variants: 2 common variants located in the *CFH* and the *C2/CFB/SKIV2L* loci—rs10922109 and rs116503776, respectively—and a rare variant located in the CFI gene, rs141853578 or p.Gly119Arg. However, the effects of these genetic risk scores are smaller compared with the single variant effects in the model for systemic complement activation described in Table 3.

Table 5. Association of complement-raising SNPs and haplotypes with AMD: SNP rs6685931 and haplotype H1-2 confer a risk for AMD

	Odds Ratio	Confidence Interval	*P* value
SNP rs3753396	1.031	0.839 - 1.223	0.756
SNP rs6685931	1.631	1.489 - 1.772	5.889×10^{-12}
Haplotype H3	1.015	0.911 - 1.130	0.795
Haplotype H6	0.828	0.637 - 1.075	0.135
Haplotype H1-2	1.318	1.223 - 1.420	1.382×10^{-12}

SNP = single nucleotide polymorphism.

Single variant and haplotype association analyses with age-related macular degeneration were performed for the 1548 individuals from the discovery cohort. Haplotype analyses were based on chi-squared tests that compared the frequency of the analyzed haplotypes in patients versus controls.

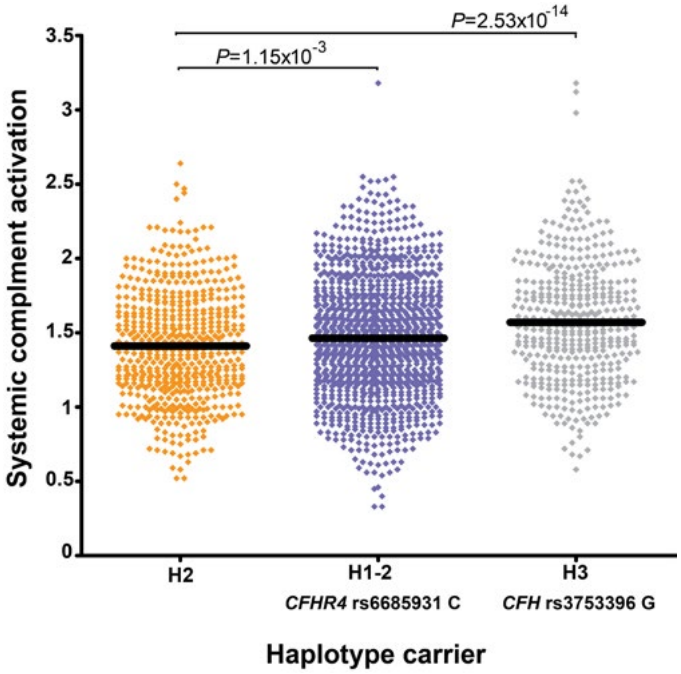

Figure 3. Graph showing complement activation levels stratified by common haplotypes across the *CFH/CFHR* locus. The AMD risk haplotype H1–2 shows high complement activation levels, and the non–AMD-associated H3–1 haplotype shows the highest. Horizontal bars indicate the mean values for each haplotype carrier group. Haplotype carriers included in the graph had a posterior probability higher than 0.75. The haplotype group colors indicate the association with AMD: orange, protective; blue, risk conferring; grey, not associated. Association analyses were carried out for the 1548 patients genotyped with exome array.

7.4 DISCUSSION

We conducted a GWAS on systemic complement activation levels, evaluating for an unbiased approach the genetic risk factors involved in the activation of this essential component of the immune system. We identified and replicated 2 common variants, rs3753396 and rs6685931, that lead to higher systemic complement activation levels independently of age, sex, AMD disease status, triglycerides, and BMI. These 2 variants were included in a model for systemic complement activation, which explained 18.7% of its variability.

The SNP rs3753396 (c.2016A→G, p.Gln672Gln) is a coding, synonymous variant located in exon 14 of the *CFH* gene, and therefore this variant, or the linked causal variant(s), may regulate complement activation levels through FH. Factor H is a key negative regulator of the AP and the amplification loop of the complement cascade, which is expressed constitutively in the liver and locally by other cell types, such as retinal pigment epithelial and endothelial cells.[35,36,37] Evidence to support the theory that rs3753396 exerts an effect on complement activation through FH comes from genetic studies on other diseases. The SNP rs3753396 has been reported to be associated with atypical hemolytic uremic syndrome, known to be caused by mutations in *CFH*.[38,39] Moreover, reduced susceptibility to meningococcal disease also has been associated with rs3753396. Meningococcal disease is caused by Neisseria meningitides, which binds FH to avoid complement-mediated killing.[40] The SNP rs3753396 is in linkage disequilibrium with rs1065489, also located in *CFH* (c.2808G→T, p.Glu936Asp), which was proposed to be the causal variant for meningococcal disease based on in silico pathogenicity predictions.[41]

The SNP rs6685931 (c.59–4315T→C) is located in intron 1 of the *CFHR4* gene. Factor H related 4 (FHR-4) is a glycoprotein that, in contrast to the attenuating effects of FH, seems to promote complement activation. It binds the complement fluid-phase C3b and forms an additional AP C3 convertase (FHR4-C3bBb), which is less susceptible to FH-mediated decay.[42] However, because rs6685931 is in high linkage disequilibrium ($r2 > 0.8$) with several variants located in the *CFH* gene, either FH or FHR-4 could be responsible for the effects observed on complement activation.

We analyzed the association of genetic variants with systemic complement activation levels in a hypothesis-free manner. The results indicate that with our study design, the genetic variants with the largest effect on complement activation levels are rs3753396

and rs668593, located at the *CFH/CFHR* locus. Moreover, other previously associated variants in *CFH* and *C2/CFB* could be replicated.[18] Haplotype analysis at the *CFH/CFHR* locus revealed 2 haplotypes with stronger effects on complement activation levels compared with the individual SNPs. These findings suggest that additional variants at the *CFH/CFHR* locus play a role in the activation of the complement system. Indeed, several rare coding variants in the *CFH* gene have been shown to lead to increased complement activity.[10] Genetic variants in other genes that influence systemic complement activation levels may be uncovered with larger sample sizes that would allow for the detection of rarer variants and smaller effects. A compelling rare variant candidate that may merit further investigation is *CFI* rs141853578 (p.Gly119Arg), which was found to be nominally significant in our study. This variant has been associated previously with lower factor I levels in plasma and a lower ability to degrade C3d on the cell surface and C3b in the fluid phase.[43]

In this study, AMD was associated with systemic complement activation, which is in agreement with previous reports.[15,16,17,18,20] In our analysis, rs6685931 in *CFHR4* was associated with both systemic complement activation and AMD. Haplotype analyses were in line with these results; we observed that the complement-raising allele of SNP rs6685931 (C) was located mainly on the H1–2 haplotype, which associated with a higher risk for AMD development. Thus, this SNP and its linked haplotype could serve as a robust biomarker for complement activation in the context of AMD and could be used to identify AMD patients who would benefit most from complement-inhibiting therapies.

We noted that the rare haplotype H6 (with a frequency of 3%), also containing rs6685931, had a larger effect on complement activation levels compared with the single variant rs6685931. However, haplotype H6 was not associated significantly with AMD, probably because of statistical power limitations. Studies with larger cohort sizes may clarify the role of the H6 haplotype in AMD and may identify other rare haplotypes that associate with AMD and have larger effects on complement activation levels.

Strikingly, the genetic variant that was associated most strongly with systemic complement activation, rs3753396 in *CFH*, and its main haplotype (H3) did not associate with AMD. However, the SNP rs3753396 and haplotype H3 have been described to confer risk for atypical hemolytic uremic syndrome development. Atypical hemolytic uremic syndrome is a complement system-related disease that leads to

systemic thrombotic microangiopathy and renal endothelial injury.[39,44] This finding suggests that the effect of the haplotypes may be different systemically compared with the AMD disease site, possibly through a tissue-specific effect of the genetic variants. Consequently, systemic complement activation may not always reflect complement activation in the disease tissue, and therefore, it may not be the most appropriate measure for AMD studies. Genetic biomarkers such as SNP rs6685931 and haplotype H1–2 are robust markers that, together with the C3d-to-C3 ratio, could serve as biomarkers for complement activity studies in AMD. This is supported by a recent study demonstrating that complement activation levels in aqueous humor are higher than in plasma samples of AMD patients.[45] As a consequence, the effect of rs6685931 and H1–2 on local complement activation may be even larger than the effect seen on systemic levels.

Our results also could further the understanding of other complement-related diseases, as well as be used in the context of personalized medicine involving FH supplementation therapy and other complement-targeting therapies.[46,47,48] Besides N. meningitidis, a number of bacteria, fungi, parasites, and viruses bind FH to avoid elimination by the alternative pathway of the complement system.[49] Also, some cancer cells express FH to avoid being targeted by the immune system.[50,51,52] Other FH-related diseases for which our results may be of interest include hemolytic uremic syndrome, atypical hemolytic uremic syndrome, encephalomyelitis, atherosclerosis, insulin resistance, immunoglobulin A nephropathy, Alzheimer's disease, cisplatin nephropathy, as well as severe dengue, for which variants in the *CFH* gene have been shown to be protective.[53]

In conclusion, we identified 2 common variants located at the *CFH/CFHR* locus, rs3753396 and rs668593, which strongly influence systemic complement activation levels. Moreover, our haplotype studies suggest that other genetic variants in the *CFH/CFHR* locus influence systemic complement activation. Genetic and nongenetic factors identified in this and other studies explain up to 18.7% of the variability in systemic complement activation levels. The common variant rs6685931 in *CFHR4*, and its associated haplotype H1–2, could be used, together with other environmental factors as well as rare genetic variants, to select AMD patients who would benefit from complement-inhibiting therapies.

7.5 SUPPLEMENTARY DATA

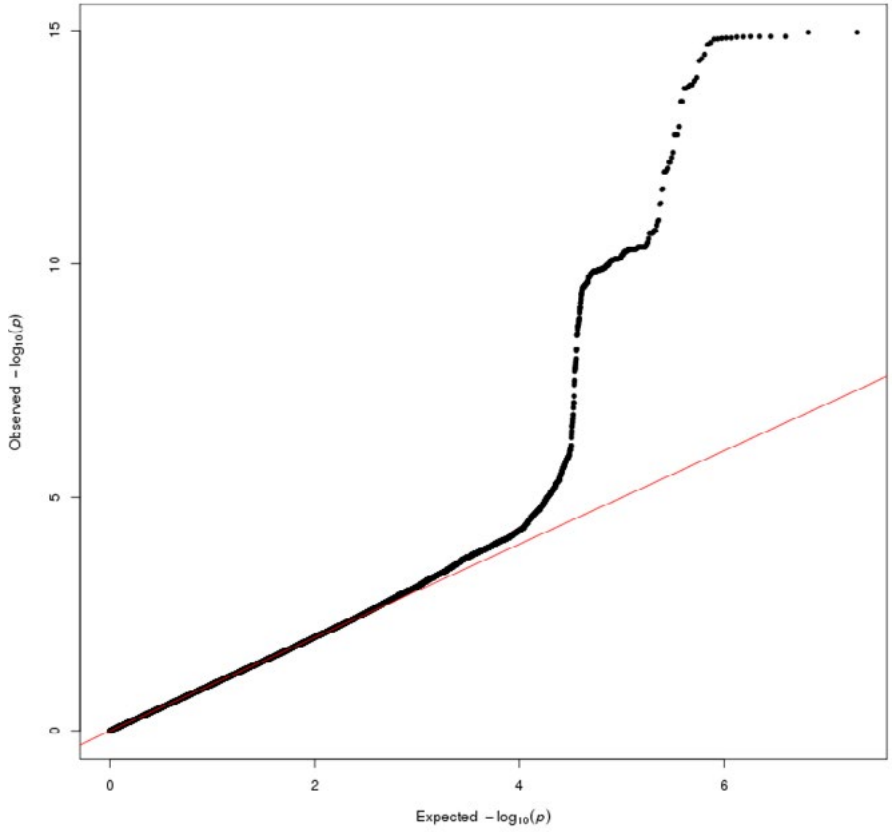

Figure S1. Q-Q plot of the GWAS on systemic complement activation levels. Shown as black dots are the observed P-values (–log10(P)). Lack of population stratification was confirmed by a genomic inflation factor (λ) for the trend of 0.999.

Table S1. General linear model for systemic complement activation levels including environmental factors

	β	SE (β)	*P*-value	R^2
Age (years)	0.003	0.001	0.002	0.010
Sex (female)	-0.025	0.018	0.155	0
Disease status (AMD)	0.074	0.017	6.700×10^{-5}	0.013
BMI (kg/m2)	-0.012	0.002	8.635×10^{-8}	0.034
Triglycerides (mmol/l)	-0.130	0.010	5.723×10^{-39}	0.101
HDL cholesterol (mmol/l)	0.015	0.026	0.545	0.024
Clinic (University Hospital of Cologne)	-0.064	0.016	6.800×10^{-5}	0.005

BMI=Body mass index, HDL=High-density lipoprotein, SE=standard error.
R^2=0.141 (adjusted R^2=0.138).

Table S2. Association of variants identified in previous studies

Gene	Genetic variant	Study	Allele	Complement activation measurement	Direction of the effect	*P*-value	This study					
							Allele	AF	*P*-value	β	SE (β)	Replicated / Not replicated
CFH	rs800292	Hecker et al., 2009	G	Ba	+	$7.06x10^{-6}$	G	0.79	$2.545x10^{-4}$	0.058	0.016	Replicated
CFH	rs800292	Hecker et al., 2009	G	C3d	+	0.0013	G	0.79	$2.545x10^{-4}$	0.058	0.016	Replicated
CFB	rs4151667	Hecker et al., 2009	T	Ba	+	$3.86x10^{-6}$	T	0.95	$3.48x10^{-6}$	0.151	0.032	Replicated
C2	rs9332739	Hecker et al., 2009	G	Ba	+	$1.98x10^{-6}$	G	0.96	$1.63x10^{-6}$	0.157	0.033	Replicated
C3	rs2230199	Reynolds et al., 2009	G	C5a	+	0.04	G	0.22	0.044	-0.034	0.017	Not replicated
ARMS2	rs10490924	Reynolds et al., 2009	T	C5a	+	0.02	T	0.30	0.417	0.012	0.014	Not replicated

AF=Allele frequency, SE=Standard error.

Table S3. Meta-analysis of discovery and replication cohorts identifies two signals at the CFH/CFHR locus associated with systemic complement activation levels: analysis adjusted for AMD status

			Discovery cohort (n= 1,548)		Replication cohort (n=697)[§]		Meta-analysis (n=2,245)[¶]	
Lead variant (MA)	Chr.: Position*	Gene†	β (SE)	*P*-value	β (SE)	*P*-value	β (SE)	*P*-value
rs3753396 (G)	1:196695742	*CFH*	0.145 (0.018)	$1.316x10^{-15}$	0.128 (0.027)	$1.717x10^{-6}$	0.1396 (0.015)	$6.741x10^{-21}$
rs6685931 (C)	1:196867233	*CFHR4*	0.061 (0.014)	$9.561x10^{-6}$	0.030 (0.015)	0.043	0.046 (0.010)	$3.928x10^{-6}$

MA=Minor allele, Chr=Chromosome, MAF=Minor allele frequency, SE=Standard error.

*Chromosome and chromosomal position according to the NCBI RefSeq hg19 human genome. †Closest gene to the lead variant. §Replication cohort for rs6685931 consisted of 686 individuals. ¶Meta-analysis for rs6685931 was performed in a total of 2234 individuals.

Table S4. Predicted haplotypes across the *CFH/CFHR* locus

Haplotype	rs3753394	rs529825*	rs800292*	rs3766404	rs1061170	rs203674	rs3753396†	rs1065489†	rs6685931
H1-2 (rs6685931-C)	C	G	G	T	C	G	A	G	C
H2	C	A	A	T	T	T	A	G	T
H3	T	G	G	T	T	T	G	T	T
H4	C	G	G	C	T	T	A	G	T
H5	T	G	G	T	T	T	A	G	T
H1-1 (rs6685931-T)	C	G	G	T	C	G	A	G	T
H6	T	G	G	T	T	G	A	G	C
H7	T	G	G	T	C	G	A	G	C
H8	T	G	G	C	T	T	A	G	T

*SNPs in linkage disequilibrium (r^2=1). †SNPs in linkage disequilibrium (r^2=1).
Haplotypes are coded as in Hageman et al., 2005. If two different sub-haplotypes based on the extra allele in SNP rs6685931 were found, the Hageman haplotypes were recoded as 1 or 2.

Table S5. Association of the 52 AMD variants with systemic complement activation levels

Variant*	AMD risk-increasing allele	Locus name	AF†	β†	SE (β) †	*P*-value†	*P*-value†‡
rs10922109	C	*CFH*	0.629	0.089	0.013	$5.389x10^{-11}$	$1.406x10^{-9}$
rs570618	T	*CFH*	0.421	-0.002	0.013	0.876	0.629
rs121913059§	T	*CFH*	-	-	-	-	-
rs148553336	T	*CFH*	0.995	-0.128	0.097	0.186	0.219
rs187328863	T	*CFH*	0.042	0.028	0.036	0.445	0.668
rs61818925	G	*CFH(CFHR3/CFHR1)*	0.637	0.015	0.015	0.306	0.187
rs35292876	T	*CFH*	0.016	0.031	0.052	0.550	0.648
rs191281603	G	*CFH*	0.01	0.041	0.088	0.639	0.578
rs11884770	C	*COL4A3*	0.738	-0.022	0.015	0.146	0.176
rs62247658	C	*ADAMTS9-AS2*	0.417	0.007	0.013	0.616	0.475
rs140647181	C	*COL8A1*	0.018	-0.092	0.057	0.109	0.106
rs55975637	A	*COL8A1*	0.129	-0.015	0.020	0.473	0.427
rs10033900	T	*CFI*	0.495	0.004	0.014	0.762	0.664
rs141853578	T	*CFI*	0.003	0.292	0.133	0.028	0.042
rs62358361	T	*C9*	0.014	0.025	0.057	0.668	0.742
rs114092250	G	*PRLR/SPEF2*	0.975	-0.046	0.045	0.300	0.297
rs116503776	G	*C2/CFB/SKIV2L*	0.872	0.059	0.020	0.003	0.005
rs144629244	A	*C2/CFB/SKIV2L*	0.010	0.067	0.065	0.300	0.360
rs114254831	G	*C2/CFB/SKIV2L*	0.242	-0.01	0.015	0.508	0.426
rs181705462	T	*C2/CFB/SKIV2L*	0.008	-0.008	0.075	0.921	0.877
rs943080	T	*VEGFA*	0.510	-0.026	0.013	0.056	0.075
rs1142	T	*KMT2E/SRPK2*	0.371	0.002	0.014	0.883	0.948
rs7803454	T	*PILRB/PILRA*	0.184	-0.01	0.017	0.574	0.484
rs79037040	T	*TNFRSF10A*	0.518	0.011	0.013	0.411	0.338
rs10781182	T	*MIR6130/RORB*	0.301	-0.008	0.015	0.594	0.701
rs71507014	G	*TRPM3*	0.421	-0.005	0.014	0.732	0.724
rs1626340	G	*TGFBR1*	0.793	-0.009	0.017	0.598	0.468
rs2740488	A	*ABCA1*	0.740	-0.009	0.015	0.555	0.403
rs12357257	A	*ARHGAP21*	0.238	-0.001	0.016	0.927	0.915
rs3750846	C	*ARMS2/HTRA1*	0.297	0.01	0.014	0.468	0.866
rs3138141	A	*RDH5/CD63*	0.210	0.046	0.021	0.030	0.045
rs61941274	A	*ACAD10*	0.013	0.003	0.072	0.971	0.980
rs9564692	C	*B3GALTL*	0.727	-0.008	0.015	0.618	0.506
rs61985136	T	*RAD51B*	0.619	0.007	0.014	0.620	0.541
rs2842339	G	*RAD51B*	0.095	0	0.023	0.994	0.975
rs2043085	T	*LIPC*	0.616	0.029	0.014	0.036	0.039
rs2070895	G	*LIPC*	0.800	-0.03	0.017	0.071	0.062
rs5817082	C	*CETP*	0.745	0.017	0.016	0.284	0.402
rs17231506	T	*CETP*	0.326	-0.001	0.014	0.924	0.723
rs72802342	C	*CTRB2/CTRB1*	0.929	0.003	0.028	0.914	0.932
rs11080055	C	*TMEM97/VTN*	0.503	0.015	0.013	0.246	0.206
rs6565597	T	*NPLOC4/TSPAN10*	0.391	0.003	0.015	0.824	0.862
rs2230199	G	*C3*	0.224	-0.034	0.017	0.044	0.060
rs147859257	G	*C3*	0.006	0.098	0.089	0.270	0.385
rs12019136	G	*C3(NRTN/FUT6)*	0.958	-0.012	0.035	0.740	0.689
rs67538026	C	*CNN2*	0.556	0.001	0.015	0.960	0.900
rs429358	T	*APOE*	0.883	0.022	0.021	0.311	0.294
rs73036519	G	*APOE(EXOC3L2/MARK4)*	0.697	0.012	0.015	0.453	0.477
rs142450006	TTTTC	*MMP9*	0.858	0.009	0.020	0.667	0.705
rs201459901	T	*C20orf85*	0.948	-0.024	0.030	0.425	0.444
rs5754227	T	*SYN3/TIMP3*	0.871	-0.003	0.020	0.895	0.796
rs8135665	T	*SLC16A8*	0.210	0.003	0.016	0.853	0.973

AF= Allele frequency, SE= Standard error.

*Location of the SNP as in Fritsche et al., 2016, †Refers to the AMD risk-increasing allele, ‡Analysis adjusted for AMD disease status, §Variant not found in the study cohort.

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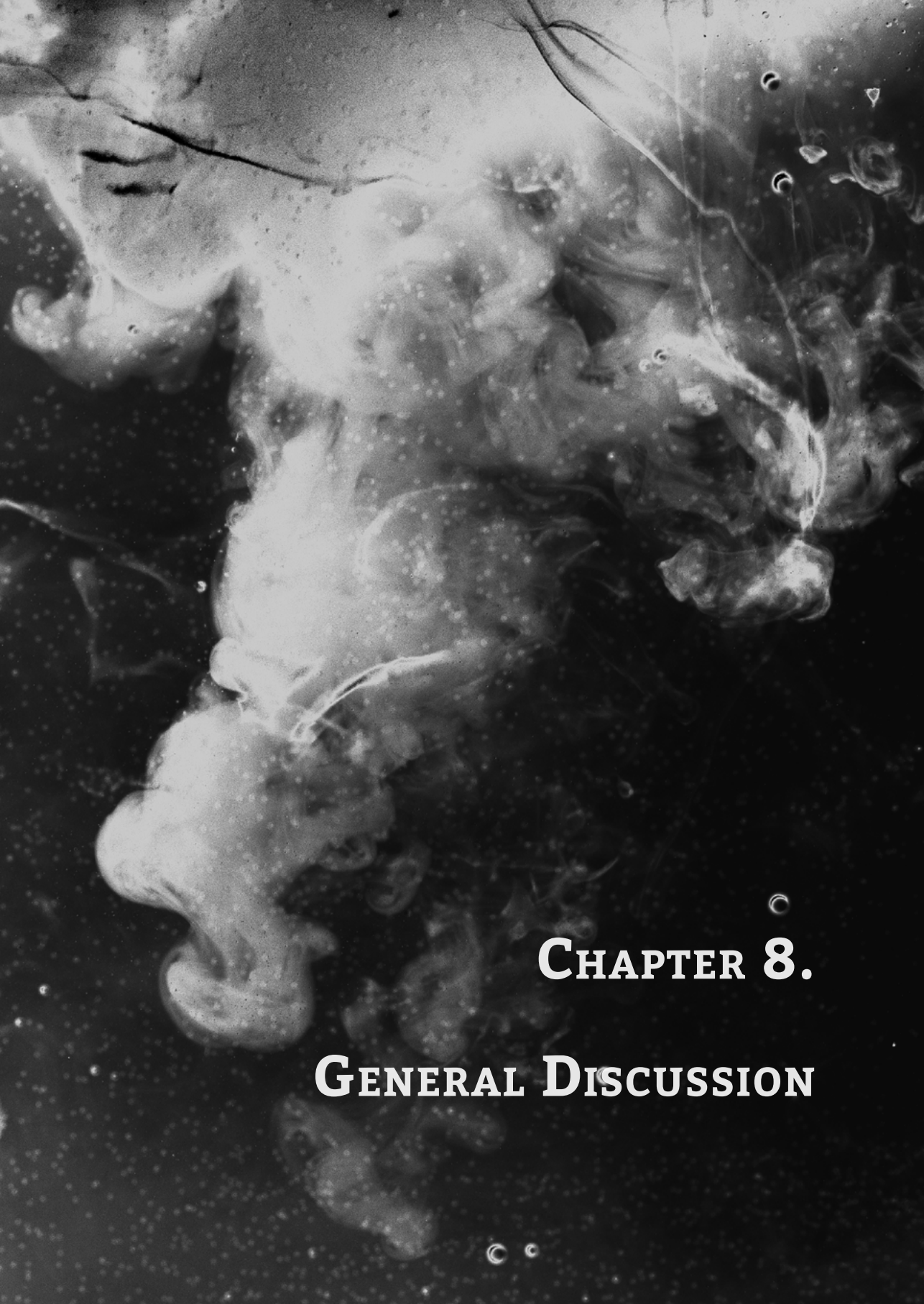

Chapter 8.

General Discussion

GENERAL DISCUSSION

The overarching aim of this thesis was to better understand the molecular drivers of age-related macular degeneration (AMD). In this final chapter the obtained results are discussed in a broader perspective, considering the current state of the AMD research field.

AMD is a multifactorial disease for which the pathogenesis is not yet fully understood. Outside influences like diet, smoking, physical fitness and sun exposure interact with a person's genetic blueprint and the combination of these factors establishes a certain predisposition to develop AMD in a person's lifetime. Multiple biological pathways and physiological networks have been associated with the disease, such as the complement system, lipoprotein homeostasis, extracellular matrix biology, angiogenesis and altered redox states [1]. Because of strong genetic and physiological evidence, the complement system has been the focus of intense investigation and has also been a target for potential novel therapies for AMD in the last several years.

8.1 WHY INVESTIGATE FACTORS INVOLVED IN COMPLEMENT ACTIVATION?

Since the discovery of higher complement activation levels in patients with AMD compared to controls [2-5] we have focused our attention on understanding which factors influence complement activation in the context of AMD.

Besides generating novel scientific insights into how complement activation is regulated, in recent years, these research questions became increasingly relevant because of their translational implications. Several clinical trials using complement inhibitors in AMD have been initiated to investigate their potential in slowing down disease progression (Table 1). Unfortunately, these inhibitors were largely unsuccessful in phase II and/or III trials.

In part, the disappointing outcomes of most of these trials may be explained by the fact that complement activation levels can vary considerably among individuals. In line with this observation, initially we hypothesized that patients who have higher levels of complement activation may benefit more than others from therapies targeting an

Table 1. Clinical trials for complement system modulators in AMD.

Drug	Target	Status
Eculizumab,	Complement component 5	Phase II has been completed and it did not decrease the growth rate of GA significantly [6]
Lampalizumab	Complement factor D	Two phase III clinical trials. One did not meet its primary endpoint of reducing mean change in GA lesion area [7] while the results from the other have not been shared yet.
Avacincaptad pegol	Complement component 5	Phase II/III clinical trial has started.
Tesidolumab, LFG316	Complement component 5	Phase II clinical trial completed but the results have not been published yet.
CLG561	Properdin	Phase II clinical trial has started
POT4	Complement component 3	Phase II/III terminated by management NCT01603043
APL2	Complement component 3	Phase II clinical trial has started

over-active complement system. In order to identify the most suitable patients for complement-inhibiting therapies, we would first need to gain a better understanding in the factors that influence complement activation.

The complement activation cascade is triggered through classical, mannose-binding lectin or alternative pathways, which were described in the introduction of this thesis. Importantly, the amplitude of the response, in part, is influenced by genetic and non-genetic factors, although the exact nature of these influencing factors were not known, nor was it clear how these factors interacted. We followed several strategies to uncover which genetic factors are central to complement regulation, which are covered in section 8.2, while some of the non-genetic factors, like interactions with lipids/lipoproteins are discussed in section 8.5.

8.2 WHICH GENETIC FACTORS INFLUENCE COMPLEMENT ACTIVATION?

Before we started our studies, there was evidence that at least parts of the complement system are under genetic control. Bayesian variance components models had previously shown that serum levels of certain complement components like C3 and C4 have 40-45% heritability [8,9]. Therefore, we hypothesized that complement activation levels might also be genetically driven. To uncover the genetic variants that influence complement activation we undertook several approaches. In Chapter 4 a targeted approach was used to investigate the effect of AMD-associated SNPs on complement activation.

The selected SNPs for this study came from GWAS studies that had identified several complement-related variants to be associated with AMD [10]. To test whether these SNPs would also explain the observed elevation in complement activation in patients we performed univariate and multivariate association analyses. Significant associations with complement activation were found for three SNPs in the *CFH* gene (rs1410996, rs800292 and rs12144939), one SNP in *CFB* (rs4151667) and two SNPs in the *C3* gene (rs6795735 and rs2230199). All SNPs in the *CFH* and *CFB* genes showed lower complement activation levels compared to the reference alleles, while the SNPs in *C3* were associated with higher values. Including the SNPs in a model together with age, smoking status, gender and disease status explained only about 6.7% of the total variability in complement activation (Chapter 4).

Several other studies have also evaluated the effect of genetic variation on complement activity; however, all these studies were restricted to a limited number of SNPs. [3,4] In chapter 5 we identified a novel complotype, which is in essence a combination of minor functional genetic variants that can accumulate to large effects because they are all part of the same cascade. The complotype we reported was associated with both AMD and systemic complement activation. Both this complotype and other previously described complotypes were composed of three SNPs. [11] This is very little considering the large number of SNPs in complement genes that have been found to be associated with AMD. In an ideal situation, all of them would be tested in one model, however this is impractical, because of the enormous amount of possible genotype combinations that would require very large patients datasets that are not currently available. The notion of using a complotype has a number of other limitations. A major shortcoming of this targeted approach is that it does not take into account all the other variants

that might occur in the genome that could exert some influence on the overall activity of the complement cascade. A secondary concern is that genetic functional variants in the complement system can either increase or decrease complement activation and unexpected interactions between these variations are not accounted for.

To tackle this issue, in Chapter 7 we performed the first GWAS in an effort to identify in an unbiased manner the genetic loci that determine systemic complement activation levels. This approach revealed two independent signals that were significant at a genome-wide threshold level ($P < 1 \times 10^{-8}$). Both signals were situated in the *CFH-CFHR* gene cluster on chromosome 1. Including the top SNPs (rs3753396 in *CFH* and rs6685931 in *CFHR4*) from these loci in a model together with age, AMD disease status, body mass index, triglycerides and the previously identified SNPs, explained 18.7% of variability in complement activation, which is a considerable percentage for any biological trait.

This model may be even further improved if other complement influencing factors are identified and included, such as oxidative stress levels as we discussed in Chapter 2. Also, reducing variability itself within the actual measurement of complement activation would improve the accuracy of the model. At this time, the model is based on measurements taken from patients and controls at one time point and does not take into account variation through the day nor any clinical or subclinical signs of infection. In part, these issues can be resolved by using multiple complement activation measurements for each subject over an extended period of time and also enrolling fasting patients could potentially reduce some of the variability. An important next step is to validate this model in other cohorts that have the same variables available so that it can be determined how reproducible these observations are and to what extent they can be used in clinical practice.

8.3 IS AMD DRIVEN BY LOCAL OR SYSTEMIC COMPLEMENT ACTIVATION?

Systemic complement activation is associated with AMD, but it might not be the main driver of the disease. It is not clear yet whether AMD is a local disease manifestation of a systemic phenomenon or the result of locally produced factors within the aging macula. A recent study highlighted that, in contrast to monogenic eye disorders where

the genes are often uniquely expressed in the retina, the genes involved in AMD are mostly ubiquitously expressed throughout the body. [12] Also, the genetic loci identified in AMD are shared between several other complex diseases such as cardiovascular, autoimmune and neurological disorders among others [13], indicating that the genetic risk for AMD is not limited to an eye phenotype.

In contrast to this, there is evidence to suggest that risk factors associated to the disease are more pronounced in the eye itself compared to the systemic circulation. One example comes from a recent study that revealed significantly increased levels of complement activation products in aqueous humor of neovascular AMD patients compared to controls [14] whereas in plasma measurements these differences were far less obvious. Additionally, upregulation of complement pathway genes have been observed in transcriptome profiles of AMD retinal tissue versus control, demonstrating active local transcription, rather than systemic influx. [15] Also, Bruch's membrane/choriocapillary extracts from advanced AMD eyes were found to have elevated CFB, C3, C3a, and CFD levels compared to tissue from eyes lacking macular drusen. [16] Furthermore, reductions in MCP/CD46, a cofactor for CFI-mediated cleavage of C3b and C4b, were observed in early AMD and GA donor eyes, preceding atrophy and correlating with disease severity [17,18]. Finally, only certain systemic complement proteins are able to diffuse from the choriocapillaris through the BrM into the eye. [19] Considering all of this information, future research should be focused more on local precipitating events in the eye that lead to the development of AMD.

8.4 WHY IS THE TOP SNP IN THE COMPLEMENT GWAS NOT ASSOCIATED WITH AMD?

The apparent paradox between systemic complement activation and local disease is also illustrated by the outcomes of our GWAS. The main SNP identified in the GWAS we performed and described in Chapter 7, rs3753396 located in *CFH*, is associated with the highest levels of systemic complement activation, yet has no association with AMD.

The *CFH* gene is 22 exons long and it encodes complement factor H (FH), one of the major inhibitors of the complement system. This gene has two alternative transcripts: one encodes a full-length FH which is translated from all 22 exons, and the second

encodes a shorter isoform called FHL-1, which stops after splicing of an alternative 10th exon [20]. The top SNP from the GWAS presented in Chapter 7 (rs3753396) was located in the fourteenth exon of *CFH*, making it solely part of the full-length FH. It is an adenine to a guanine change at cDNA position 2016 (c.2016A>G), which at protein level leads to a synonymous amino acid substitution at position 672 (p.Gln672=). This position is located in the 11th complement control protein (CCP) module, which together with the neighboring modules forms a compact arrangement, consistent with a hinge-like structure [21]. However, being a synonymous change makes it very difficult to explain how it affects complement activation levels. One possibility is that it is a proxy for a different variant that actually does alter protein expression or function and thus influences complement activation.

A possible explanation for the lack of association with AMD, may come from the fact that that full-length FH does not pass the blood-eye barrier, but only the truncated form (FHL-1) does. [19] Thus any SNP located exclusively in the full-length part of the protein, like rs3753396, would not influence local complement activation in the eye. Moreover, the variant located in *CFHR4* that is associated both with complement activation and with AMD is interesting for follow-up studies, since the role of FHR4 in AMD has not yet been investigated in great detail. It would be interesting to test how the FHR protein family in general and FHR4 in particular is linked to AMD in the context of what we already know of the involvement of complement components in the disease.

8.5 WHAT ARE THE INTERACTIONS OF COMPLEMENT WITH OTHER PATHWAYS?

Interestingly, we reported a number of non-genetic associations with complement activation such as age, AMD disease status, body mass index, triglyceride levels and high density lipoprotein cholesterol (HDL-C) (Chapter 6). Of particular interest is the observation that complement activation is related to factors that are modifiable in nature, in relationship with diet and exercise. Even though genetic markers are robust and have the benefit of not being dynamic, having more knowledge about how complement interacts with other biological systems such as the lipoprotein metabolism may open up avenues that in the future could lead to novel treatments that are complementary to the ones being developed now.

Besides its well known role in innate immunity, there is convincing evidence that complement is involved in metabolic events and pathologies. [22] Diseases like metabolic syndrome (MetS) and diabetes as well as cardiovascular dysfunction have been linked to complement components, such as C3 and C4. [23,24] These two complement components have been strongly linked to BMI and fat distribution, in particular through the actions of a direct degradation product of C3, C3-des-Arg, also known as acylation stimulating protein (ACP), which coordinates energy supply and fat storage. [25] In Chapter 6 we also show significant correlations between complement activation and HDL-C, apolipoprotein A1 (ApoA1) and triglycerides. Another link between complement and the lipid metabolism comes from the fact that adipocytes can synthesize alternative pathway components (C3, factor B, and factor D) and after activation, these cells can trigger the cleavage of C3 into C3b and C3a.[22,26]

Multiple types of lipoprotein classes have been linked functionally and/or structurally to the complement system. The largest and least dense particles are chylomicrons (CMs). The major cargo of CMs are triglycerides, but they are also known to carry transthyretin, which has been shown to stimulate complement C3 (C3) and ACP/C3desArg synthesis in a dose-dependent manner. [27] At the other end of the density spectrum in the lipid metabolism, HDL-C is connected with the complement system in multiple ways. Firstly, apolipoprotein components of HDL-C can regulate complement activity. ApoA1 and ApoA2, the two main components of HDL-C, were shown to inhibit complement-mediated lysis. [28] Another component of HDL-C that inhibits complement-mediated lysis is ApoJ, also known as clusterin. [29] Finally, ApoE can also be present on HDL and it influences complement activation by binding to FH, one of main complement regulators. [30]

In addition to this, proteomic studies have shown that HDL-C carries multiple complement proteins such as C3, complement factor C4B, Factor B, complement C5 and to a lesser extent complement C1 subcomponents and complement C2 [31]. Why lipoproteins carry complement components is not yet understood. Another proteomic study in a different lipoprotein subclass, Lipoprotein(a) (Lp(a)), also reported detection with high confidence of C3 and C4A [32]. Furthermore, specific complement components or regulators are reported to be carried by specific subsets of HDL-C. One study observed that all of the FHR1 in plasma is located in one lipoprotein fraction [33]. This lipoprotein fraction was identified to be a small subfraction of HDL-C (about 2% of APOA1 containing HDL-C) 33,34. Later, also dimerized forms of FHR4 [35] and FHR5 [36] were found to be associated with triglyceride-rich lipoproteins.

From studies done in other diseases, it is apparent that the complement protein content of the HDL-C particle is not static, but dynamic. For example, in coronary artery disease (CAD), complement complements C3 and C4 were elevated in the HDL-C from patients compared to controls, while clusterin was reduced [37]. Under the influence of statins and niacin, the composition of HDL-C could be remodeled to resemble HDL-C normally found in healthy individuals [38]. Also, in rheumatoid arthritis, changes in the complement composition of HDL-C were reported (factor B, C3 and C9 were increased) [39].

The precise function of the presence of complement components and regulators in the lipoprotein fractions is not yet fully understood, however it does reveal an intimate link between the two systems. For AMD the relevance of these findings is strengthened by the fact that drusen from eyes of AMD patients have a composition of >40% lipids, the rest being an array of complement components, TIMP3, vitronectin, β-amyloid, and apolipoproteins (E, B, A1, C-I, and C-II), plus zinc and iron ions. [40-42]

Taken together, these studies suggest that circulating lipoprotein levels and their composition and biological activity might be important in the pathogenesis of AMD.

Another pathway that has been associated with AMD and which interacts with the complement system is that of oxidative stress. Increased oxidative stress is thought to be one of the key factors in the occurrence of AMD. The high metabolic activity and high PUFA content in the membranes of photoreceptors primes the macula to high oxidative stress. [43] Malondialdehyde (MDA) is one of the reactive carbonyl compounds originating from PUFA oxidation, and its presence is often used to measure lipid peroxidation levels in blood or serum samples. [44-46] In Chapter 2 we have seen that increased systemic levels of MDA have been consistently observed in both wet and dry AMD. MDA is a highly reactive molecule that forms covalent bonds with endogenous proteins. These MDA modifications can be recognized by factors of the immune system, thus triggering a proinflammatory response by increasing the expression of the inflammation factor interleukin (IL)-8. [47] FH can neutralize the proinflammatory response generated by MDA. Interestingly in this context, the Y402H FH polymorphism, which is associated with increased risk for AMD, leads to reduced binding to MDA, thus potentially contributing to a chronic proinflammatory response.[48]

8.6 WHAT DO THESE FINDINGS MEAN FOR THE PREDICTION AND TREATMENT OF AMD?

We are entering an exciting era of personalized medicine, where rapid technological advancements in all –omics fields will transform patient care. Currently vision loss in AMD is largely irreversible. The only available treatment for the wet form of AMD, which represents only a minority of AMD cases, is the intraocular injection of inhibitors of vascular endothelial growth factor (VEGF). [49] For the majority of AMD patients that suffer from early or intermediate AMD or geographic atrophy, currently, no treatment is yet available. As has been discussed in paragraph 8.1, all phase II and/or III clinical trials investigating complement inhibitors have failed in their effectiveness.

The results obtained in Chapters 4 and 7 could help in the stratification of patient subgroups by facilitating the identification of individuals that are genetically primed towards higher complement activation levels. Such stratification has the potential of identifying patients in which complement-inhibition therapy could work. However, there are also other potential reasons why the trials have been unsuccessful to date.

A major contributing factor may be that treatment was administrated rather late in the overall development of AMD. Patients with end-stage geographic atrophy were selected and monitored for disease progression, i.e. enlarging of the atrophic area. This means that potentially irreversible damage has already occurred, and inhibiting the complement at this stage would have little to no effect. Complement activation, in the context of a full immunological response, is an early responder. Its activation and degradation products play a role in attracting immune cells which, from that point onwards, begin to take over and coordinate the immunological response in either an M1 (pro inflammatory) or M2 (anti-inflammatory) manner. [50] Thus, after years of slowly progressing from early to intermediate stages, it may well be that the actual contribution and role of the complement system is rather different in end stages. If this would be the case, then the need for good prediction models is essential and genetic predisposition analysis will become even more relevant. The discussion would then rather be whether early intervention, at the intermediate stage or even earlier is justified.

So far, only a limited number of complement components have been targeted by complement-inhibiting therapies. Choosing different targets might yield better results.

Another element that is worth considering is the fact that it is still not known whether higher levels of complement activation in AMD patients are a cause or a consequence of the disease. Mendelian randomization (MR) analysis, developed to unravel causes and consequences when multiple variables are tightly entwined, could help to shed light on this issue. MR was recently successfully implemented in AMD to show that long-term elevated levels of plasma HDL-C increase AMD risk. [51] A similar approach could be done for the relationships between complement activation levels, complement activation-associated variables and AMD disease status. The SNPs that we identified in Chapter 7, central to systemic complement activation would be excellent candidates for this type of study.

Lastly, considering the complexity of AMD and the interplay of multiple pathways associated with the disease, it is not unlikely that a combined type of intervention, aimed at more than one contributing factor rather than monotherapy, is required. In order for this to become a reality, much more detailed work needs to be done on the exact role of the implicated pathways and their interactions, across the different disease stages of AMD.

In summary, the results presented in this thesis have demonstrated that systemic complement activation is partly under genetic control and partly influenced by modifiable factors such as triglycerides and HDL. Also, this thesis demonstrates that systemic complement activation may not be the major driver of the disease as was previously thought. The results presented here embed the complement system in a tightly regulated and intertwined network of other biological systems, such as the lipoprotein metabolism. Combined, these results can be implemented to identify patients predisposed to a higher level of complement activation, and those patients could be excellent candidates for upcoming clinical trials, targeting the complement system. Moreover, the results presented here are a first step towards an even more detailed understanding of the role of complement in AMD and the interaction with other pathways. Developing these lines of research further has the potential to uncover novel therapeutic options for patients in early or intermediate stages of AMD.

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Summary

SUMMARY

Age related macular degeneration (AMD), the most common cause of blindness in the Western world, is a disease of the eye affecting the central part of the retina. AMD is a complex multifactorial disease where both genetic and environmental factors play a role in its development. In this thesis we investigated and combined genetic results with physiological measurements in AMD patients to gain a better understanding of the molecular drivers of AMD.

Chapter 1 provides the reader with a general introduction to the major subjects of this thesis.

Chapter 2 explored all available literature on potential biomarkers in AMD present in serum or in ocular fluid. The results of this exploration yielded a comprehensive overview where initially over 3600 articles, describing a plethora of compounds measured in serum, plasma, vitreous, aqueous humor and urine of AMD patients, were screened. After condensing that information, the review delivers for each compound a short description and a summary of its usefulness as a potential biomarker for AMD. We identified compounds belonging to the oxidative stress pathway, the complement system, and lipid metabolism to be the most promising biomarker candidates in AMD.

In Chapter 3 we examined genetic variants in the *C3* gene, which encodes a central component of the complement system, in order to determine if rare mutations could give a high risk for developing AMD. We identified three rare variants (Lys65Gln, Arg735Trp and Ser1619Arg) at the C3 locus that were associated with AMD in the EUGENDA cohort. However, the Arg735Trp and Ser1619Arg variants could not be replicated in the Rotterdam Study. The Lys65Gln variant was only identified in patients from Nijmegen, the Netherlands, and thus may represent a region-specific AMD risk variant.

Chapters 4 and 5 investigated the relationship of known AMD-associated single nucleotide polymorphisms (SNPs) with complement activation levels. In Chapter 4 we analyzed the association of 32 SNPs in or near 23 AMD-associated genes with serum levels of C3d/C3 as a measure of complement activation. Only SNPs in *C3*, *CFB* and *CFH* were found to influence complement activation levels.

In Chapter 5 we report on a novel complotype, which is a combination of SNPs that individually have small effects, but when they are combined, their effects amplify. This novel complotype was associated with both AMD status and complement activation levels. The complotype is composed of 2 SNPs in *CFB* and one in *CFH*.

Chapter 6 bridges the gap between genetic associations and physiological lipid levels in AMD. Additionally, we observed, for the first time, strong correlations between complement activation and lipid/lipoprotein levels. The implication of these results is that two major systems that were previously thought to act independently in AMD, could in fact work in concert in the disease.

In Chapter 7 we further advanced the work we had developed in Chapters 3, 4 and 5 by undertaking an unbiased approach to identify genetic variants that influence complement activation levels. We identified 2 loci that are independently associated with AMD at a genome-wide significance level: One in *CFH* and the other one in *CFHR4*. These findings may help to identify AMD patients who would benefit most from complement-inhibiting therapies. In this study we uncovered that the haplotypes that are most associated with systemic complement activation levels are in fact not associated with AMD, which further finetunes our understanding about the role of the complement pathway and complement activation in AMD.

Chapter 8 discusses the main findings of this thesis in a broader perspective. The entire discussion is structured as a series of questions and their potential answers. We cover topics ranging from genetic drivers of complement activation to why thus far complement inhibiting therapies have not been successful. Finally, we offer an outlook on how the results generated in this thesis could impact the research field of AMD as well as patient care.

SAMENVATTING

Leeftijdsgebonden maculadegeneratie (LMD), de meest voorkomende oorzaak van blindheid in Westerse landen, is een oogaandoening waarbij het centrale deel van het netvlies aangetast is. LMD is een complexe ziekte, veroorzaakt door een combinatie van erfelijkheid en omgevingsfactoren. In dit proefschrift hebben we onderzoek verricht naar erfelijke factoren en stoffen in het bloed, en hun onderlinge relatie, om een beter begrip te krijgen van de moleculaire aanjagers van de ziekte LMD.

Hoofdstuk 1 informeert de lezer over de algemene begrippen en grote thema's die in dit proefschrift worden besproken.

In Hoofdstuk 2 is alle literatuur op het gebied van mogelijke biomarkers in het bloed en in oogvocht verzameld, geanalyseerd en gerubriceerd. In eerste instantie zijn bijna 3600 artikelen gescreend die een grote verzameling stoffen beschreven die gemeten zijn in serum, plasma, voorste oogkamerwater, glasvocht, en urine van patiënten. Deze informatie werd samengevoegd, waarna in het overzichtsartikel elke gevonden stof kort werd omschreven, gevolgd door een samenvatting over het nut van de stof als biomarker voor LMD. We vonden stoffen die betrokken zijn bij oxidatieve stress, bij het complement systeem en de vetzuurhuishouding de meest veelbelovende biomarkers voor de LMD.

In hoofdstuk 3 deden we onderzoek naar genetische varianten in het *C3* gen en de relatie met LMD. We onderzochten met name of zeldzame varianten een verhoogd risico kunnen geven op LMD. We vonden drie zeldzame varianten (Lys65Gln, Arg735Trp en Ser1619Arg) in het *C3* gen die geassocieerd waren met LMD in het EUGENDA cohort. De associaties met de Arg735Trp en Ser1619Arg varianten werden niet bevestigd in een cohort uit Rotterdam. De Lys65Gln variant vonden we alleen in LMD patiënten uit de omgeving van Nijmegen, wat suggereert dat dit mogelijk een regiogebonden variant zou kunnen zijn.

In hoofdstukken 4 en 5 onderzochten we of bekende genetische varianten die betrokken zijn bij LMD ook geassocieerd zijn met complement activiteit. In hoofdstuk 4 testten we 32 genetische varianten in 23 LMD genen en hun associatie met serum niveaus van C3d/C3 als maat voor complement activiteit. Alleen genetische varianten in *C3*, *CFH* en *CFB* bleken complement activiteit te beïnvloeden.

Hoofdstuk 5 beschrijft een 'complotype', een combinatie van genetische varianten die elk afzonderlijk kleine effecten teweeg brengen, maar gezamenlijk een sterk effect hebben. We beschreven een nieuwe complotype dat zowel met LMD als met complement activiteit geassocieerd is. Het complotype bestaat uit twee varianten in *CFB* en een variant in *CFH*.

Hoofdstuk 6 beoogt een brug te slaan tussen genetische associaties en vetzuur niveaus in LMD. We vonden een sterke correlatie tussen bepaalde vetzuur-gerelateerde stoffen en het complement systeem. The implicatie van deze uitkomst is dat twee van de grote biologische systemen die eerder werden geassocieerd met LMD, maar als onafhankelijk van elkaar werden gezien, mogelijk samenwerken in het ziekteproces van LMD, iets wat eerder niet bekend was.

In hoofdstuk 7 onderzochten we of we, zonder vooraf bepaalde hypothese, genetisch factoren konden vinden die samenhangen met complement activiteit. We vonden twee gebieden op het genoom dicht bij elkaar die bepalend lijken te zijn voor systemische complement activiteit: Een in het *CFH* gen en een in het *CFHR4* gen. Deze kennis kan mogelijk in de toekomst worden gebruikt om LMD patiënten te selecteren voor behandeling met complement-remmende middelen in LMD. Verder vonden we dat bepaalde haplotypes, een opeenvolgende rij van genetische varianten, die sterk geassocieerd waren met complement activiteit, helemaal niet geassocieerd waren met LMD. Deze informatie scherpt onze gedachten over de rol van het complement systeem in LMD verder aan.

In hoofdstuk 8 worden de belangrijkste bevindingen van dit proefschrift samengevat en in een breder perspectief geplaatst. De discussie is gestructureerd in een aantal vragen en hun mogelijke antwoorden. We bespreken verschillende onderwerpen, zoals de genetische oorzaken van complement activiteit, en waarom behandeling met complement-remmende middelen tot nu hoe niet succesvol zijn geweest bij LMD. Tenslotte geven we een vooruitzicht in hoe de resultaten uit dit proefschrift gebruikt kunnen worden in toekomstig onderzoek naar de ziektemechanismen van LMD en hoe ze toegepast kunnen worden in de patiëntenzorg.

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About the author

ABOUT THE AUTHOR

Codruț Constantin Păun was born on 14th of February 1986 in Reșița, România. In 2005 he started studying Genetic Engineering at Banat's University of Agricultural Sciences and Veterinary Medicine from Timișoara, România from which he graduated in the summer of 2009 by obtaining an engineers diploma in the field of biotechnologies. During these years he has performed both volunteering and research assistantship work at the University's Genetics department under the guidance of Prof. Galia Butnaru.

In the fall of 2009 he came to the Netherlands as part of the Top research master's program, Molecular Mechanisms of Diseases (MMD), from which he graduated in 2011 with the title Master of Science. The same year he started his Phd program under the supervision of Prof. Anneke den Hollander, Dr. Eiko de Jong and Prof. Carel Hoyng.